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Rhipicephalus sanguineus group (Acari: Ixodida) of Western Iberia Peninsula and Africa: Mitochondrial lineages study

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Aos meus pais pelo seu amor e apoio incondicional…

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Os Agradecimentos e o Sumário não seguem as novas regras do acordo ortográfico.

As referências bibliográficas nesta dissertação estão de acordo com as normas da revista *Journal Tick and Tick-borne Diseases*.

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Sumário

As carraças ou ixodídeos (Ordem Ixodida: Classe Arachnida) são artrópodes, ectoparasitas e hematófagos obrigatórios. Estão distribuídas a nível mundial e usam como hospedeiros répteis, aves, anfíbios, mamíferos e acidentalmente o homem. São também vectores de vários agentes patogénicos, alguns com potencial zoonótico.

São conhecidas cerca de 850 espécies de carraças que se agrupam em três famílias: Nuttallielidae, Argasidae e Ixodidae. A família Nuttallielidae conta apenas com uma espécie que pode ser encontrada no continente africano. A família Argasidae conta com cerca de 170 espécies diferentes, sendo a sua principal característica a ausência de escudo dorsal, pelo que se conhecem comumente como "carraças de corpo mole". A família Ixodidae inclui cerca de 650 espécies, que dada a presença de um escudo dorsal de quitina, são conhecidas por "carraças de corpo duro",

Dentro da família Ixodidae podemos encontrar o género *Rhipicephalus*, que conta com 84 espécies com elevado interesse médico e veterinário. Dentro do género, destaca-se a espécie *Rhipicephalus sanguineus* (Latreille, 1806), cuja taxonomia tem sido um tema de debate ao longo dos últimos 50 anos, possivelmente devido à inexistência de um holótipo e da recente descoberta de inúmeras espécies crípticas nela incluídas. Devido ao grande número de outras espécies do género que também são confundidas morfologicamente com *R. sanguineus*, foi formando um complexo ou grupo de espécies designado por grupo *R. sanguineus*, que conta com, pelo menos 12 espécies nele incluídas.

Em Portugal, *R. sanguineus sensu lato* (em oposição à designação *sensu stricto*, que de momento não é aceite) encontra-se distribuido por todo o país, e é o principal vector de diversos agentes patogénicos, entre os quais *Babesia canis* (babesiose canina), *Ehrlichia canis* (ehrlichiose canina) e *Ricketsia conorii* (febre escaro-nodular).

A diferenciação das espécies de carraças é tipicamente feita com base em caracteres morfológicos, o que acarreta algumas limitações devido à grande variabilidade inter e intraespecífica presente no grupo. Dado que a correcta identificação é um factor chave para a associação entre uma espécie vectorial específica e um agente patogénico, torna-se essencial encontrar formas alternativas e mais eficazes para proceder com a sua correcta identificação. O recurso a marcadores moleculares, são tidos como eficientes, baratos e com resultados reprodutíveis para desempenhar este tipo de avaliações.

Com base nestas ferramentas, e com recurso a três marcadores moleculares mitocondriais (COI mtDNA, 16S rDNA e 12S rDNA) foi possível determinar que dentro da nossa colecção de amostras identificadas como *R. sanguineus s.l*, colhidas em Portugal e em alguns países Africanos, que existem pelo menos cinco linhagens com base no DNA mitocondrial: duas linhagens com morfologia tipo *R. sanguineus* (a linhagem temperada e a linhagem tropical), e três linhagens que apresentavam uma morfologia tipo *R. turanicus* (CEM, SeA e EM).

Os resultados obtidos permitiram determinar que as amostras colectadas em Portugal pertencem à linhagem temperada enquanto que a maioria das amostras colectadas em países africanos pertencem à linhagem tropical. Dentro da linhagem temperada foi possível evidenciar a existência de subclades, correlacionados com as diferentes áreas geográficas onde as amostras foram colectadas. Com os resultados obtidos foi também possível evidenciar que as duas subespécies reconhecidas de *R. evertsi* (*R. evertsi evertsi* e *R. evertsi mimeticus*) podem ser afinal duas espécies distintas, sendo assim sugeridas para reavaliação biosistemática. É também corroborado pelos nossos resultados que apesar da morfologia tipo *R. turanicus* ter sido evidenciada existir na Península Ibérica, os indivíduos que a apresentam são molecularmente identificados como pertencentes à linhagem *R. sanguineus* temperada. Desta forma, as linhagens *R. turanicus* não foram ainda evidenciadas como existentes na área, e mais estudos serão necessários para clarificar a plasticidade fenotípica existente neste clade.

Por fim, foi possível detectar, com recurso a marcadores moleculares (16S rDNA), que algumas das amostras recolhidas de animais selvagens, que se sabia previamente estarem doentes, estavam infectadas com bactérias da família Anaplasmataceae. Este resultado é fortemente indicativo de que carraças da linhagem temperada foram o vector da bactéria.

Palavras chave: *Rhipicephalus sanguineus sensu lato*, zoonoses, filogenia, marcadores moleculares, linhagens mitocondriais.

Abstract

Rhipicephalus sanguineus sensu lato, commonly known as “brown dog tick”, is a three-host tick that parasitizes mainly dogs and occasionally humans. It is worldwide distributed and an vector of several important zoonosis. Its taxonomic status is currently under debate since there is already evidence that it includes several cryptic species. To clarify further this issue, we applied three mitochondrial markers (COI mtDNA, 16S and 12S rDNA) to perform a phylogenetic analysis using samples collected in Portugal and Africa. We were able to establish five different mitochondrial lineages for *R. sanguineus s.l.:* two *R. sanguineus*-like and three *R. turanicus*-like lineages, which were correlated to their geographic origin.

Samples collected in Portugal were identified as belonging to the temperate lineage, and the main African collected ones belonged to the tropical lineage. Furthermore, it was possible to observe a set of subclades within these clades, suggesting an ongoing process of divergence. It is also suggested that the two *R. eversti* currently recognized as subspecies should be considered independent species, thus we suggest a biosystematics re-evaluation of both entities. Moreover, although *R. turanicus*-type-morphology is found on the Iberian Peninsula, molecular results do not support the existence of *R. turanicus* mitochondrial lineages on the area. Further studies, based on phenotypic plasticity presented by the clade, should be conducted to clarify this matter.

Finally, we detected a molecular trace (based on 16S rDNA mitochondrial region) of Anaplasmataceae family of bacteria in ill hosts collected ticks, suggesting a recent infection transmitted by a *R. sanguineus*-like tick.

Key words: *Rhipicephalus sanguineus sensu lato*, zoonosis, molecular markers, phylogeny, mitochondrial lineages.

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List of Abbreviations

COI: Cytochrome c oxidase I

mtDNA: Mithoncondrial deoxyribonucleic acid

rDNA: Ribosomal deoxyribonucleic acid

rRNA: Ribosomal ribonucleic acid

DNA: Deoxyribonucleic acid

ITS2: Internal transcribed spacer 2

PCR: Polymerase chain reaction

CERVAS: Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens

RIAS: Centro de Recuperação e Investigação de Animais Selvagens da Ria Formosa

BS: Bootstrap values

ML: Maximum Likelihood

NJ: Neighbour-Joining

CEM: Central East Mediterranean

EM: East Mediterranean

SeA: Southern east African

TBD: Tick Borne Diseases

TBP: Tick Borne Pathogens

SC: Subclade

CCHF: Crimean Congo Hemorrhagic Fever

s.s: *Sensu stricto*

s.l: *Sensu lato*

1. Background

1.1 Ixodids ecological and biological features

Ticks, belong to class Arachnida, order Ixodida, and are divided on three major families, Argasidae, Ixodidae and Nuttalliellidae (only includes a South African single species). On total there are around 800 tick species worldwide divided in about 18 genera. The Argasidae family, is known as "soft ticks" contrarily to Ixodidae that are "hard ticks", due to the presence of a sclerotized scutum (Estrada-Peña and De La Fuente, 2014; Silva et al., 2006). Other morphological and ecological characteristics differentiate these two families, as their way to ingest blood and also the duration of a blood meal. Argasidae ticks are more often associated to birds, and the Ixodidae family include the species with major economic, medical and veterinary importance, and therefore more studies are found on the literature associated to this group/family (Estrada-Peña, 2015).

Ixodids, or ticks, are included on the arthropods that are able to interact with vertebrates. The main interactions are parasitic relationships, where the parasite, in this case the tick, takes metabolic and physiologic advantages of their hosts. It is reported that mammals and their parasitic arthropods have co-evolved over the years (Kim, 1985).

Ticks are strict hematophagous ectoparasites of domestic animals, livestock and wildlife. Moreover ticks are able to act as vectors of several pathogenic agents, such as virus, bacteria and protozoa, some of them with zoonotic potential (Estrada-Peña, 2015) which makes them the second worldwide vectors of human diseases, behind mosquitos (Eskezia, 2016; Papa et al., 2016). Tick-borne diseases (TBD) are the most common vector-borne diseases in Europe (Jahfari et al., 2016). In terms of numbers, 10% of the 800 species of ixodids around the world carry tick-borne pathogens (TBP) that have zoonotic potential (Fuente et al., 2017).

Ixodids may be associated with economic losses. They are the most important ectoparasites of livestock, causing weight and blood losses, dermatitis, meat and milk quality, which decreases the commercial value of the hosts. To humans some TBD diseases are associated with high morbidity rates, and on extreme cases even death. This brings a public health issue where both vectors and hosts have to be monitored in order to prevent major morbidity or mortality cases (Jongejan and Uilenberg, 2004).

The life cycle of ixodids counts with four development stages: egg (the only inactive stage), larvae, nymph and finally adult (the active stages). Every transition within the active stages is characterized by a moult and also by a necessity of a blood meal from a host. Blood meals may last from a few minutes (in case of larvae and nymphs) to days or weeks (in adult stage) and this meal provide them the necessary energy for the moult or oviposition. It is also during blood meals that ticks become able to infect their host with pathogenic agents (Estrada-Peña, 2015).

The number of hosts that ticks needs in order to complete their development varies from one to three. One host ticks, moult and feed in the same host, in all the active stages. Two host ticks, both larvae and nymph feed on the same host, without drop on the ground between moults, but nymphs detach from the host and moult on the ground and then the adults must find another suitable host. Finally, there are ticks in which the larvae feed in one host, then drop out and moult to nymph that as to use another host, and the same for that adult, therefore they are three host ticks. When a tick tries to find a host, it rises their front legs in a behaviour called questing. The duration and the intensity of these questing moments have deep implications for the epidemiology of TBD (Estrada-Peña and De La Fuente, 2014).

Questing behaviour ends when the tick finds a suitable host to its next meal. Both the development stage and abundance or diversity of hosts on the area will interfere on host choice. Immature stages as larvae and nymphs tend to feed on small animals as birds or rodents; the adults easily can feed on larger mammals, even wild or domestic (Estrada-Peña, 2015; Estrada-Peña and De La Fuente, 2014). There is no concordance within the studies done until today in regard to established strict relationships between ticks and key hosts, ticks seem to be generalist parasites, which increases their potential as vector of TBD (McCoy et al., 2013).

Once a tick finds a host, they find an appropriate and sheltered spot where they insert the mouthparts, after that first step the "cement" is release which immediately solidifies in contact whit the host skin, allowing the ticks attachment. Then a series of complex events sequence begins, mainly originated in the salivary glands of the tick, which will allow to lysis the cells surrounding the feeding area, sustaining the blood flows and host's immune response evasion (Estrada-Peña, 2015; Estrada-Peña and De La Fuente, 2014).

It is also via saliva that the TBP is inoculated on the host. Saliva molecules implicated in potentiating TBP transmission are being used as a potential vaccine candidate to apply either on animals and humans. However, there were no concrete progresses that allow us to point out to their effectiveness on TBD control (Wikel, 2013).

After matting, engorged females detach from the host and look for a protected site to lay thousands of eggs, after which they died. High humidity is essential to ensure egg survival and larvae hatching. The total time for a tick to complete their life cycle is variable among the existing species. Some species might reach four development stages in one year, contrasting to other that take three or four years from egg to adult. This last situation is more likely in colder climates (Estrada-Peña, 2015).

This introduces us to a concept summarised by Estrada-Peña and Fuente (2014), ticks "phenology". Phenology stands for the influence that both microclimate and hosts availability/ diversity has on tick abundance and seasonality. The combination between these factors will determine in each time of the year ticks pass to each stage of their life cycle. Temperature seems to have more influence as regulator of development rates and mortality. These two factors depend on the losses of water, which are regulated by the relative humidity and air saturation. During the winter, low temperature prevents fast development, which progresses slowly until the rise of temperatures in the spring. In the spring, large numbers of active ticks appear in the vegetation at temperate regions as a consequence of the many moulting ticks driven by the rise of temperature. Temperature and water availability influences are not the same in every geographical region. In cold climates, temperature will probably play the most significant role in the regulation of phenology together with the photoperiod while in dry regions, water availability becomes a key variable. For these reasons we can find ticks worldwide despite that, the tick abundance will be affected by the tick phenology of the species present on each country (Estrada-Peña and De La Fuente, 2014).

1.2 Taxonomic background

Ticks belong to phylum Arthropoda, class Arachnida, subclass Acari, superorder Parasitiforme, order Ixodida, superfamily Ixodoidea that is divided in three families: Argasidae, Ixodidae and Nuttalliellidae.

Hoogstraal and Aeschlimann (1982) first established the evolutionary relations between the hard ticks families. They divided them in two major groups: Prostriata (Ixodinae; *Ixodes*) and Metastriata. The Metastriata group comprises 4 subfamilies that are, in order of divergence from the original stem, Amblyomminae (*Amblyomma* and *Aponomma*), Haemaphysalinae (*Haemaphysalis*), and the sister clades Hyalomminae (*Hyalomma*) and Rhipicephalinae. The subfamily Rhipicephalinae encompasses 9 genera, among them *Rhipicephalus* (75 species), *Dermacentor* (30 species), *Margaropus* (3 species), and *Boophilus* (5 species). All of these data came from biological, morphological and host choice datas (Fig. 1).

Black and Piesman (1994) corroborates the results of Hoogstraal and Aeschlimann using the 16S rDNA marker to establish a phylogenetic tree. However, in this study some new insights emerged: Amblyomminae and Argasidae are not monophyletic, Haemaphysalinae appears within Amblyomminae, Hyalomminae cames within Rhipicephalinae.

To help the clarification of the relations between the Hyalomminae and Rhipicephalinae subfamilies, Murrell *et al.* (2001) lead a molecular study with 12S rDNA marker. From this study some conclusions were made: (1) the genus *Rhipicephalus* is paraphyletic with respect to the genus Boophilus, (2) the genus *Dermacentor* is paraphyletic with respect to the genus *Anocentor*, and (3) some subgenera of the genera *Hyalomma* and *Rhipicephalus* are paraphyletic with respect to other subgenera in these genera. Also in this work the authors suggested the exchange of the species from genus *Boophilus* to be included on the genus *Rhipicephalus*.

Phylogenetic studies can also provide us another type of information, like divergence times between different taxa. Because significant gaps in the fossil record exist, the divergence times of important chelicerate groups remain uncertain. They are possible to estimate from a phylogenetic analysis of mitochondrial or nuclear sequences, but such divergence estimates require the generation of a phylogenetic tree with strong support for all clades. The oldest fossil record for the order Parasitiformes goes back only to Cretaceous (late Mesozoic Era) about 90–94 million years ago (Jeyaprakash and Hoy, 2009).

Using complete mitochondrial genome of 25 chelicerate taxa, Jeyaprakash and Hoy (2009) were able to suggest that orders and classes of spiders, scorpions, mites, and ticks diversified in the late Paleozoic, much earlier than previously reported from fossil date estimates. Ticks from Ixodidae family arose in the late Permian and early Triassic, while the prostriate hard ticks (*Ixodes*) and soft ticks (Argasidae) started diversifying between the late Triassic and early Jurassic. It appears that metastriate hard ticks (*Rhipicephalus, Amblyomma* and *Haemaphysalis*) diversified much later, between the late Jurassic and the early Cretaceous.

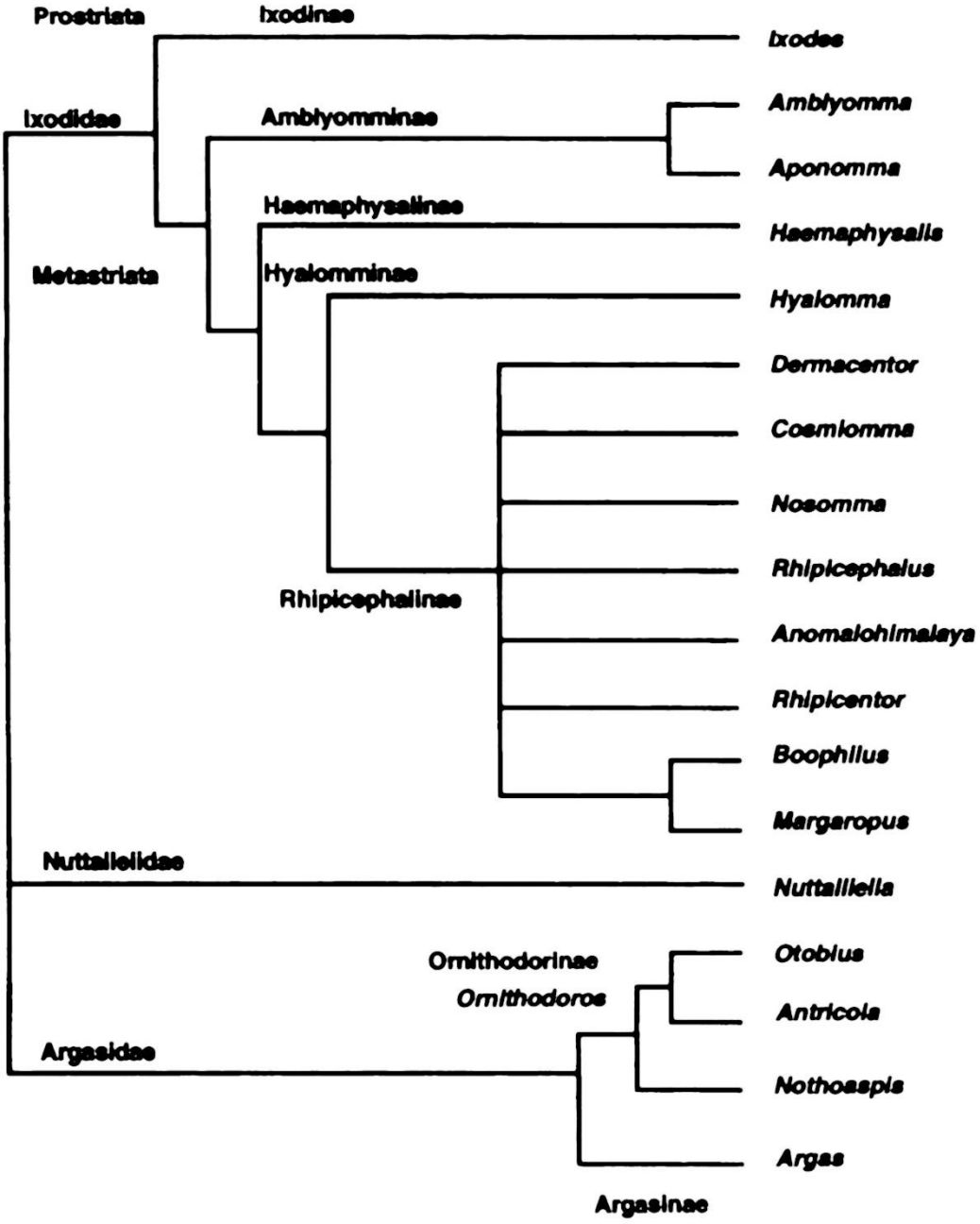

Figure 1: Phylogeny of families, subfamilies and genera of soft and hard ticks. Propose by Hoogstraal and Aeschlimann (1892) and it was based on biological, morphological and host choice data. (Black and Piesman (1994))

1.3 The *Rhipicephalus* genus

Within the Ixodidae family, which counts with about 650 species worldwide (Silva et al., 2006), exists the *Rhipicephalus* genus, counting with 84 species with special medical and veterinary interest (Latrofa et al., 2013). The major number of species from this genus can be found in Africa, but also they are well represented on Europe (Mehlhorn, 2014). In this genus we found the most reported parasite on livestock, *R. microplus* (McCoy et al., 2013) and also the most world spread parasite of domestic dogs, *Rhipicephalus sanguineus* (Latreille,1806).

Mainly in *Rhipicephalus* genus the species are three host ticks, but for example, *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888) is an one host tick and *Rhipicephalus evertsi evertsi* (Neumann, 1897) and *Rhipicephalus bursa* (Canastrini and Fanzago, 1878) are two host ticks (Mehlhorn, 2014).

On this genus it is possible to separate a various number of group of species based on morphological similarities: *R. sanguineus, R. simus, R. follis, R. capensis, R. pravus, R. appendiculatus* and *R. evertsi* (Walker et al., 2000). However, it is necessary to be considered that these genus complexes and their associated species are not consensually accepted (Camicas et al., 1998).

In regard to host specificity, it is not easy to establish key relationships between ticks and their hosts. Host specificity can be defined as the extent to which a parasite taxon is restricted in the number of host species used at a given stage in the life cycle. Thus, highly host-specific parasites have one host species, and specificity declines as the number of suitable host species increases (Nava and Guglielmone, 2013).

Rhipicephalus spp. ticks, mainly parasite mammals but also can be found in birds, reptiles and amphibians, suggesting a none strict preference for the species of the host (Gray et al., 2013; Hornok et al., 2017; Latrofa et al., 2013).

Two contrasting hypotheses have been developed about host specificity in ticks. The first assumes that host specialisation was a result of the evolution of ticks' morphological characters in order to be better adapted to the host and therefore is based on the idea of coevolution between ticks and tetrapods. Nava and Guglielmone (2013) assume that phenotypic variations in mouthparts and coxae are the result of adaptation to a particular group of hosts, which lead to a high host specificity. In contrast, other authors raised an alternative hypothesis where the importance of ecological specificity is highlighted. According to this opinion, adaptation to a particular habitat is more relevant for tick evolution than adaptation to a particular host (Nava and Guglielmone, 2013). Other author, McCoy (2013) point out that not only the number of hosts used by the ticks might be considered as well as their fitness on each host species, but that type of study has not been yet performed. None of the theories are fully accepted and among the little studies done for this issue, suggesting an extremely complex relation between environment, tick and host, which need to be clarified in future studies.

A more recent work done by Esser *et al.* (2016) with ticks from a strict geographic region, tries to connected and describe each tick species they can find on each host species. They aim to explore the quantitative range of hosts for each tick they found. The results allow them to establish that ticks are specific to hosts until order and family level, which shows the major potential that ticks have for several hosts. *Rhipicephalus* genus in that study was mainly associated with order Carnivora.

Human are not preferential tick hosts, they become accidental hosts, by having contact with infected animals and just in cases where the tick doesn't have any other available host, humans become a more suitable host (Fuente, 2008).

Some morphological features distinguished this genus from the remaining ones (Fig. 2), in that family. The differential morphological features are: hypostome and palps short and, the basis capituli is usually hexagonal, festoons presence and, in the males, adanal plates shape. With the exception of four species (*R. pulchellus*, *R. maculatus*, *R. dux* and *R. humeralis*), they are inornate (the adults do not have a colour pattern on the scutum) (Walker et al., 2000).

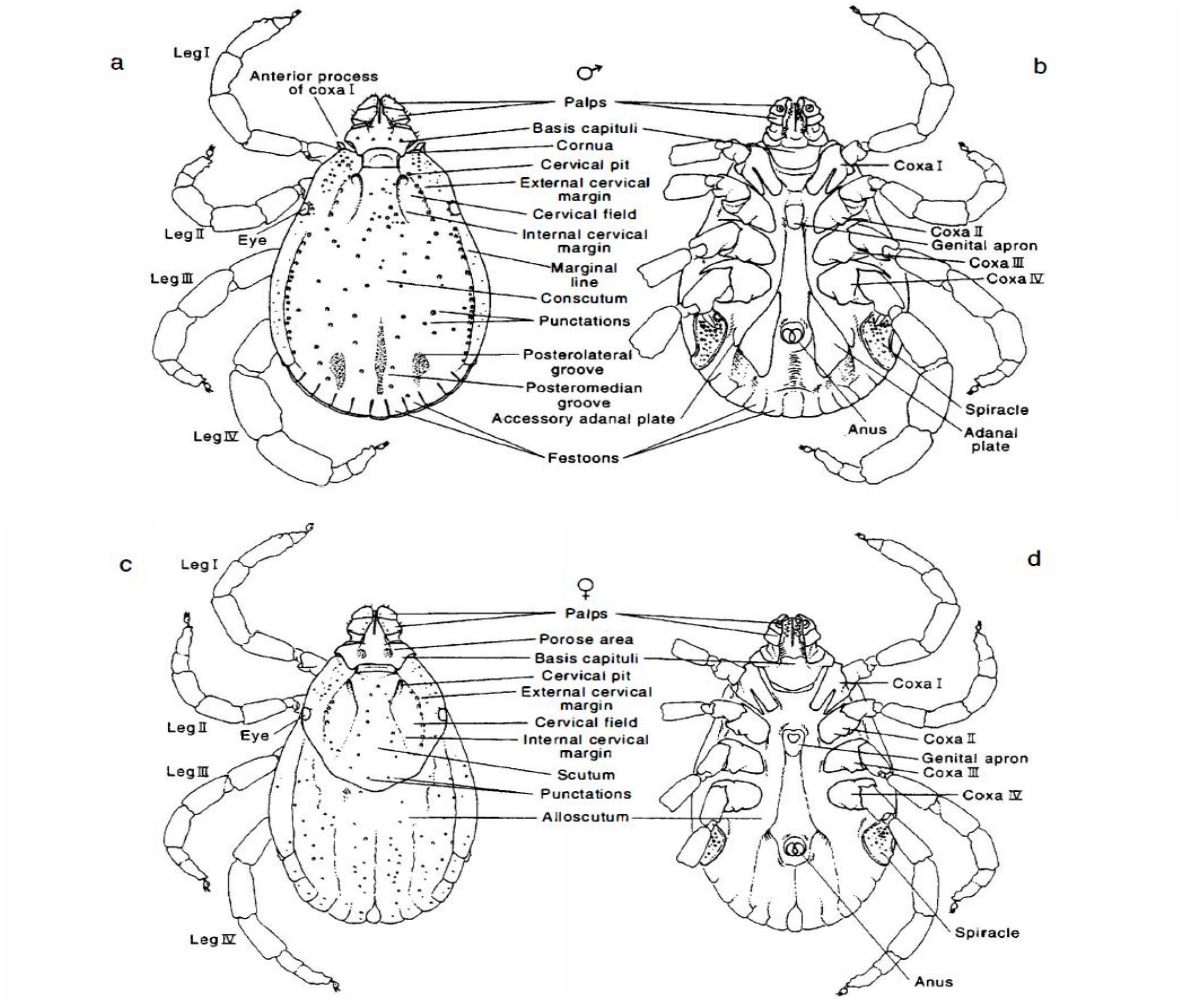

Figure 2: *Rhipicephalus* spp. mature morphological features. Male: (a) dorsal view; (b) ventral view. Female: (c) dorsal view; (d) ventral view. (Adapted from Walker *et al.* 2000)

1.4 *Rhipicephalus sanguineus* group of species issue

In 1806, Andrè Latreille describes *Ixodes sanguineus* as "*Sanguineus, punctatus, postice lineolis tribus impressus, dorso antico macula nulla thoracica, distincta*", which stands for "blood red, punctate, posteriorly with three impressed lines; no distinct thoracic spot anterodorsally", and the main area of distribution in France and therefore it was considered a Palearctic species. However Koch in 1844 transfers *Ixodes sanguineus* to the *Rhipicephalus* genus, and this become the type specimen of that same genus, but this holotype, the *R. sanguineus sensu stricto (s.s)*, has somehow been lost, and it is no longer available to be observed (Dantas-Torres and Otranto, 2015; Latrofa et al., 2013; Nava et al., 2015).

Rhipicephalus sanguineus (Fig.3), is commonly called “brown dog tick” or “kennel tick”. This species of tick has probably evolved as a parasite of carnivores in warm climates and after the domestication of the dog it as has become able to parasitize both human and canids, over a wide range of habitats. Due to many human activities, they become the most widespread of all ticks with a cosmopolitan distribution (Gray et al., 2013). Species preference for warmer climates will be affected by global warming that will be able to induce the expansion of the geographical range of *R. sanguineus* (Hornok et al., 2017).

In terms of specific habitats, they are able to use endophilic and exophilic habitats in both urban and rural environment. They are active all the year and capable to establish up to four generations per year, exhibiting passive or active host-seeking behaviour. *R. sanguineus* is a three host tick, and dogs are the preferential host on three active stages of their life cycle (Hornok et al., 2017).

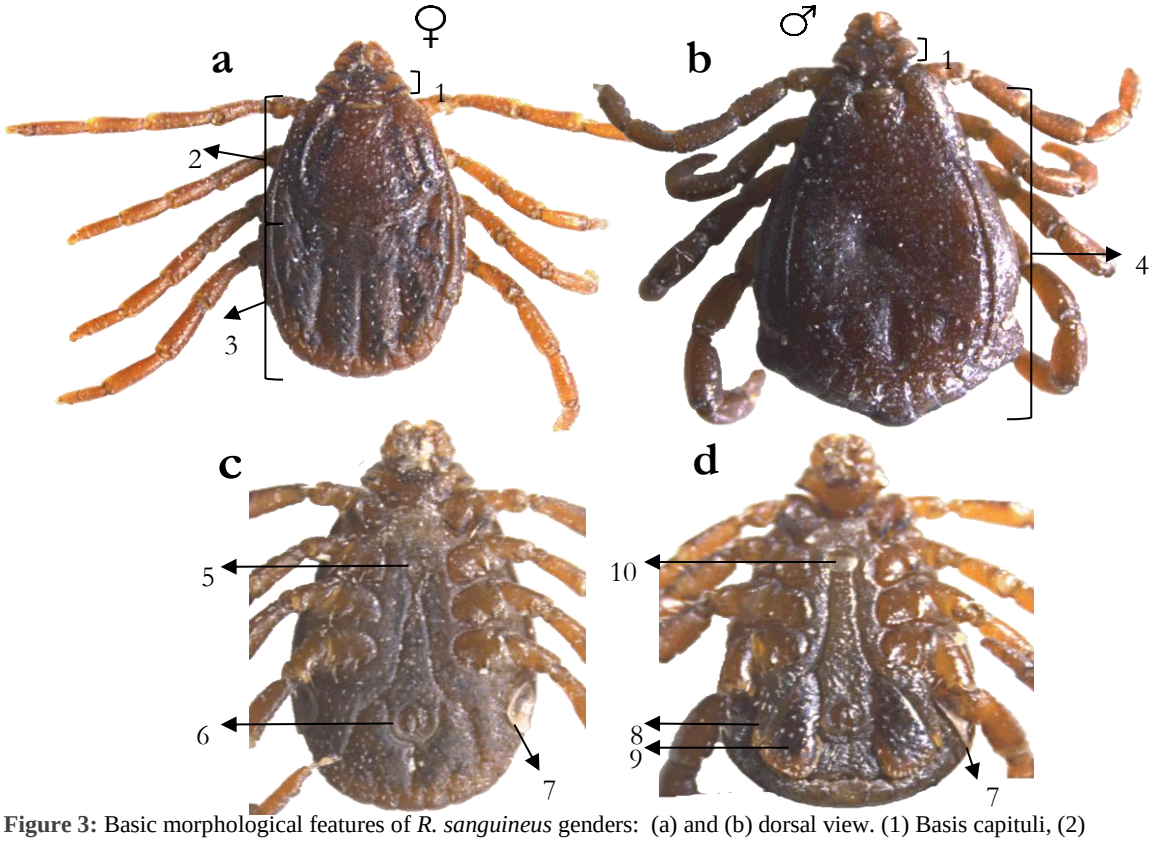

Figure 3: Basic morphological features of *R. sanguineus* genders: (a) and (b) dorsal view. (1) Basis capituli, (2) Scutum, (3) Alloscutum, (4) Conscutum (scutum united with alloscutum in the *R. sanguineus* adult males); (c) and (d) ventral view. (5) Genital aperture, (6) Anus (in the same area in both genders), (7) Spiracular area (it differs in form between the two genders), (8) Accessory adanal plates, (9) Adanal plates, (10) Spermatheca growing area. (Adapted from Coimbra-Dores (2014))

The taxonomic status of *R. sanguineus sensu lato* (*s.l.*), which implicates all the species morphologically similar to *R. sanguineus*, is still in debate, due to the lack of a holotype specimen and even due to the major morphological variety on the group is difficult to set boundaries on each species (Hekimoğlu et al., 2016; Nava et al., 2015).

The number of species belonging to the *R. sanguineus* group are variable among authors, Camicas *et al.* (1998) includes 17 species, whereas Walker *et al.* (2000) lists just 10 species and more recently Nava *et al.* (2015) counts 12 species.

Acording to Nava *et al.* (2015) the 12 species that compose this genus are: *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus sulcatus* (Neumann, 1908) , *Rhipicephalus rossicus* (Yakimov and Kohl-Yakimov, 1911), *Rhipicephalus schulzei* (Olenev, 1929), *Rhipicephalus pumilio* (Schulze, 1935), *Rhipicephalus pusillus* (Gil Collado, 1936), *Rhipicephalus turanicus* (Pomerantzev, 1940), *Rhipicephalus leporis* (Pomerantzev, 1946), *Rhipicephalus guilhoni* (Morel and Vassiliades, 1963), *Rhipicephalus moucheti* (Morel, 1965), *Rhipicephalus bergeoni* (Morel and Balis, 1976) and *Rhipicephalus camicasi* (Morel, Mouchet and Rodhain, 1976).

Many misidentifications are made within this group, consequence of the morphological variety of each species. An example of this issue is the constant confusion between *R. sanguineus* and *R. turanicus.* They are morphologically very similar, and that kind of similarity doesn't fall within the known range of morphological variety of the species group. Later on there were found features that fit on the normal morphological diversity and therefore seems to be able to distinguish them, but the molecular studies done on that specimens, prove that they were genetically indistinguishable by that time (Gray et al., 2013).

There are other topics on the group that are still on discussion as the sister species, *R. pumilio* and *R. rossicus* (Dumitrache et al., 2014; Mihalca et al., 2015), and also some re-descriptions are being made as for example the re-description of *R. camicasi* (Estrada-Pena et al., 2016).

Despite all the findings, the taxonomy status of this species is far from resolved. Along this line, a consensual re-description of *R. sanguineus s.s.* and a description of the other(s) species under this name are required, followed by an exhaustive worldwide revision of this species complex. However, morphological variations within the same genetic strain of *R. sanguineus* are quite common, which is the main current taxonomic issue (Dantas-Torres et al., 2013; Oliveira et al., 2005). Levin *et al.* (2012) and Gray *et al.* (2013) drew attention to the need of studies addressing morphology, genetic and biological aspects, considering variations of these ticks over a large geographical range.

1.5 *Rhipicephalus sanguineus* lineages

Over the last decade, some molecular and morphological studies started to indicate that what was known as *R. sanguineus s.l.* could be represented by more than one species, suggesting that the taxon *R. sanguineus* would be composed by at least two morphologically and genetically distinct lineages in the Neotropical regions (Dantas-Torres et al., 2013; Moraes-Filho et al., 2011; Nava et al., 2012).

The first insights came out with Oliveira *et al.* (2005), who showed the existence of significant differences between *R. sanguineus s.l.* ticks from Brazil (Jaboticabal, State of San Paulo) and Argentina (Rafaela, Province of Santa Fe) especially in some morphological characters related to body size, shape of the genital pore and morphology of the sensory structures.

Those differences were then evidenced molecularly by Szabó *et al.* (2005) where they found a high genetic divergence between ticks from Brazil and Argentina after comparing mitochondrial 12S rDNA sequences. They also reported a reproductive incompatibility between both tick strains.

Taking on that results, and with a collection from many different Neotropical countries, Moraes-Filho *et al.* (2011) proposed a so-called "southern lineage," located in temperate localities (Argentina, Uruguay, Chile, Italy, and south Brazil), and a "northern lineage," located in tropical and subtropical localities (Brazil, Paraguay,

Colombia, South Africa, Mozambique, and northern Argentina). Those lineages where based on both molecular and morphological data and appears to be related with the geographic origin of each specimen.

After that, Nava *et al.* (2012) observed these same lineages in the Southern Cone of South America. The authors affirm that the southern lineage is principally associated to temperate and cold localities from Argentina, Chile and Uruguay, while the northern lineage is distributed in Paraguay and in tropical areas of Argentina. The northern lineage is closely related to the *R. sanguineus s.l.* ticks present in tropical areas of America, from Brazil to southern Mexico. On top of that the authors point out that southern and northern lineages are closely related to *R. sanguineus s.s.* from Western Europe (France and Italy) and Africa (South Africa and Mozambique), respectively.

Also in that year Levin *et al.* (2012) lead a study to observe crossbreeding with ticks from different countries and lineages. Using ticks from North America, Israel and Africa, they showed the existence of reproductive barriers between the *R. sanguineus* collected in North America and Israel with the ones from Africa. Suggesting the African ones are significantly distant from the other two populations, being probably a different taxon. These results corroborate the two lineages hypothesis.

Dantas-Torres *et al.* (2013) also recognized these lineages in ticks collected in several countries from four continents, Europe, Asia, Africa and Oceania. This study uses both morphological and genetic data and it reveals that the so-called northern lineage (tropical countries) includes ticks from all continents and that both lineages are not monophyletic.

Joining to all these data, Zemtsova *et al.* (2016) lead a study where they relate climatic factors to *R. sanguineus* lineages geographic distribution. The authors conclude that tropical clade of *R. sanguineus s.l.* occupies areas with the annual mean temperature >20 °C, whereas the temperate clade is present in areas with the annual mean temperature < 20 °C. Once more it is suggested that ticks from these two closely related phylogenetic clades are adapted to different environmental conditions.

Based in these genetic data, it was suggested that exist at least two different lineages under the *R. sanguineus s.l* name, adapted to different climes and environments (Dantas-Torres et al., 2013; Dantas-Torres and Otranto, 2015).

1.6 Ticks and diseases in Portugal

Portugal is the most western region of Europe. The climate is considered oceanic along the littoral and Northern inlands, and Mediterranean in the Southern mainland. In summertime the annual average temperature varies between 16°C-26°C and 3°C-14°C in winter (Caeiro, 1999).

Currently the list of Portuguese ixodid hard ticks comprises twenty species: *Dermacentor marginatus* (Sulzer, 1776), *Dermacentor reticulatus* (Fabricius, 1794), *Haemaphysalis hispanica* (Gil Collado, 1938), *Haemaphysalis inermis* (Birula, 1895), *Haemaphysalis punctata* (Canestrini and Fanzago, 1878), *Hyalomma lusitanicum* (Koch, 1844), *Hyalomma marginatum* (Koch, 1844), *Ixodes acuminatus* (Neumann, 1901), *Ixodes bivari* (Dias, 1990), *Ixodes canisuga* (Johnston, 1849), *Ixodes frontalis* (Panzer, 1798), *Ixodes hexagonus* (Leach, 1815), *Ixodes ricinus* (Linnaeus, 1758), *Ixodes simplex* (Neumann, 1906), *Ixodes ventalloi* (Gil Collado, 1936), *Ixodes vespertilionis* (Koch, 1844), *Rhipicephalus (Boophilus) annulatus* (Say, 1821), *Rhipicephalus bursa* (Canestrini and Fanzago, 1878), *Rhipicephalus pusillus* (Gil Collado, 1938), and *Rhipicephalus sanguineus s.l* (Latreille, 1806). Among the most common species we find, *R. sanguineus*, *R. pusillus* and *R. bursa* (Santos-Silva et al., 2011).

Some authors also report the existence of *R. turanicus* as part of Portuguese list of ticks (Caeiro, 1999; Silva et al., 2006), although its existence in the area is still under debate (Coimbra-Dores et al., 2016; Dantas-Torres et al., 2017; Santos-Silva et al., 2011).

Misidentifications problems and uncertainty associated to the *R. sanguineus* and *R. turanicus* sister species identification is still a topic under debate Specimens morphologically classified as *R. turanicus* prove to be genetically indistinguishable from *R. sanguineus*, suggesting that they are a single species with a high morphological variability (Dantas-Torres et al., 2017; Santos-Silva et al., 2011). Recent molecular studies performed with ticks from all the territory, suggested once more that *R. turanicus* does not occur in Portugal (Dantas-Torres et al., 2017).

R. sanguineus s.l had been reported in all Portuguese mainland. Adults were often collected from the vegetation and from several domestic and wild mammals (carnivores, ungulates, insectivores, lagomorphs). Nymphs can be found on carnivores, insectivores, ungulates and rodents, while larvae were often found on carnivores and insectivores.

Other less reported hosts as humans and European hedgehog are becoming more suitable for this species in our country (Santos-Silva et al., 2011).

R. sanguineus s.l may be a main vector of *Anaplasma platys*, *Babesia canis* and *B. vogeli* and *Ehrlichia canis* to dogs. Furthermore, to humans, *Rickettsia conorii* strains (Malish, and Israeli spotted fever rickettsia) and *Ri. massiliae* are the main TBD associated to *R. sanguineus* (Santos-Silva et al., 2011; Silva et al., 2006).

Due to the possibility that each lineage or even population of *R. sanguineus s.l.* could have different vectorial and transmission capacities of TBP, it is extremely important to public health to clarify which group have which capacity, and where they are distributed. Since identification methods of the vector are also dependent of this clarification, this issue became one of the most studied problems in the field.

1.7 Molecular markers as solution for accrue species identification

Due to the zoonotic potential of some TBD, alternatives for correct and accurate identification are needed and on the last decade molecular identification has been a growing solution (Beati and Keirans, 2001; Black and Piesman, 1994; Latrofa et al., 2013; Lv et al., 2013). Studies suggest that different tick species carry different TBP, accurate species identifications are crucial, as they represent the basis for the establishment of effective programs to monitor tick populations, as well as to develop sustainable control and epidemiological response strategies against TBD (Dantas-Torres and Otranto, 2015; Walker et al., 2000).

Deoxyribonucleic acid (DNA) barcoding is an increasingly taxonomic method that uses a short genetic sequences of DNA to identify a particular species (Lv et al., 2013).

To be consider a precise genetic marker it must full fill some criteria: (1) should be sufficiently variable to discriminate among all species, but conserved enough to be less variable within than between species, (2) it should be standardized with the same DNA region used for different taxonomic groups, (3) it should be extremely robust with highly conserved priming sites and (4) their length should not exceed 800 bp to facilitate amplification and sequencing (Lv et al., 2013).

Cytochrome c oxidase I (COI) is considered as the preferred barcoding marker for animals. As part of mitochondrial DNA (mtDNA), which is highly conserved in organisms, makes this region a suitable marker to the species discrimination (Latrofa et al., 2013; Lv et al., 2013). The widespread use of mitochondrial DNA for phylogenetic and population genetic studies results from a relatively high mutation rate and the apparent simplicity of mitochondrial maternal inheritance compared to the nuclear DNA (Dantas-Torres et al., 2013)

Others mitochondrial markers, as 12S and 16S ribosomal ribonucleic acid (rRNA), joining nuclear markers such as internal transcribed spacer 2 (ITS2) and 18S rDNA, have also proved to be effective on molecular identification tasks. Among these 16S and 12S rRNA, stands out for best results on species-specific identifications on several *Rhipicephalus* spp. (Dantas-Torres et al., 2013; Latrofa et al., 2013; Lv et al., 2013).

In opposition, 18S rDNA seems to be very conservative within this genus and the results are more usefully on higher taxonomic levels distinctions, such as genus level (Lv et al., 2013).

2. Objectives

Based on COI, 16S and 12S rDNA markers, we aim primarily to determinate the phylogenetic lineage, of some western Iberia and Africa collected *Rhipicephalus* ticks, and if their phylogenetic variability was influenced by host diversity.

More specifically, we aimed to answer the following questions:

- Which molecular markers were more effective to amplify *Rhipicephalus* species mitochondrial DNA regions;
- If morphological clades differentiation was corroborated by the phylogenetic results;
- If *Rhipicephalus* phylogenetic lineages were associated to the geographical distribution;
- If host choice was associated to different phylogenetic lineages;

3. Materials and methods

3.1 Tick collection

For this study, a total of 66 ticks were used, 37 of them collected in Portugal, 28 collected in African countries, and one from Italy by courtesy of Dantas-Torres (Table 1).

Of the samples collected in Portugal, 12 came from the CERVAS (Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens) and 10 from RIAS (Centro de Recuperação e Investigação de Animais Selvagens da Ria Formosa) wildlife recovery centres. Five RIAS samples came with the information that the hosts-specimens were admitted due to illness. The remaining samples collected in Portugal and all the African collected ones are part of the collection of the Instituto Superior de Agronomia da Universidade de Lisboa (Lisboa, Portugal). All samples were conserved in 70% ethanol.

3.2 Morphological identification

All the specimens were morphological identified by F Rosa and MJ Coimbra-Dores using of conventional identification keys and descriptions (Coimbra-Dores et al., 2016; Dias, 1994; Papadopoulos et al., 1992; Walker et al., 2003, 2000) (Table 1).

Table 1: Tick collection information. (-) stands for information that was not available. (M) male; (F) female

Voucher	FCUL Lab Code	Area/Local	Sex	Source	Morphological identification
SF 3003	**SF1**	São Facundo	M	Vegetation	*R.turanicus*
SC 3005	**P2**	Samora Correia	M	Dog	*R.turanicus*
CR 1536	**CR 5**	Caldas da Rainha	F	Dog	*R.turanicus*
CR 1543	**CR 8**	Caldas da Rainha	M	Dog	*R.turanicus*
CR 1563	**CR 10**	Caldas da Rainha	F	Dog	*R.turanicus*
CR 1551	**CR 13**	Caldas da Rainha	F	Dog	*R.turanicus*
S 3096	**ST2**	Santarém	F	Cat	*R. sanguineus group*
S 3097	**ST3**	Santarém	F	Cat	*R. sanguineus group*
S 3100	**ST4**	Santarém	M	Domestic cow	*R. sanguineus group*
S 3101	**ST5**	Santarém	F	Domestic cow	*R. sanguineus group*
S 3104	**ST6**	Santarém	M	Sheep	*R. sanguineus group*
S 3105	**ST7**	Santarém	F	Sheep	*R. sanguineus group*
S 3106	**ST8**	Santarém	M	-	*R. sanguineus group*
Maf 3082	**MF1**	Mafra	M	Wolf	*R. sanguineus group*
Maf 3083	**MF2**	Mafra	M	Wolf	*R. sanguineus group*
PV/CE1/14	**CE1**	Viseu	F	Fox	*R. sanguineus group*
PG/CE2/11	**CE2**	Guarda	F	Fox	*R. sanguineus group*
PV/CE3/10	**CE3**	Viseu	F	Fox	*R. sanguineus group*
PG/CE4/11	**CE4**	Guarda	F	Fox	*R. sanguineus group*
PG/CE5/11	**CE5**	Guarda	M	Fox	*R. sanguineus group*
PG/CE7/11	**CE7**	Guarda	M	Fox	*R. sanguineus group*
PG/CE8/11	**CE8**	Guarda	M	Fox	*R. sanguineus group*
PG/CE9/11	**CE9**	Guarda	M	Weasel	*R. sanguineus group*
PG/CE10/11	**CE10**	Guarda	F	Weasel	*R. sanguineus group*
PG/CE11/13	**CE11**	Guarda	M	Hedgehog	*R. sanguineus group*
PG/CE12/13	**CE12**	Guarda	M	Hedgehog	*R. sanguineus group*
PG/CE13/13	**CE13**	Guarda	M	Hedgehog	*R. sanguineus group*
PF/RI3/16	**RI3**	Faro	M	Fox	*R. sanguineus group*
PF/RI4/16	**RI4**	Faro	F	Fox	*R. sanguineus group*
PF/RI5/16	**RI5**	Faro	M	Fox	*R. sanguineus group*
PF/RI6/16	**RI6**	Faro	M	Fox	*R. sanguineus group*
PF/RI7/16	**RI7**	Faro	M	Fox	*R. sanguineus group*
PF/RI9/16	**RI9**	Faro	M	Fox	*R. sanguineus group*
PF/RI11/16	**RI11**	Faro	F	Fox	*R. sanguineus group*
PF/RI10/16	**RI10**	Faro	M	Fox	*R. sanguineus group*
PF/RI12/16	**RI12**	Faro	F	Fox	*R. sanguineus group*
PF/RI17/16	**RI17**	Faro	M	Hedgehog	*R. sanguineus group*
GB 3006	**G3**	Guinea-Bissau	-	Domestic cow	*R. sanguineus group*
GB 3007	**G4**	Guinea-Bissau	-	Domestic cow	*R. sanguineus group*
MOC 3035	**M1**	Mozambique	-	-	*R. sanguineus group*
MOC 3036	**M2**	Mozambique	-	Imbabala	*R. kochi*

Table 1 (Countinued): Tick collection information. (-) stands for information that doesn't was available. (M) male; (F) female

Voucher	FCUL Lab Code	Area/Local	Sex	Source	Morphological identification
MOC 3037	**M3**	Mozambique	-	Vegetation	*R. simus*
MOC 3068	**M10**	Mozambique	-	-	*Rhipicephalus sp.*
MOC 3072	**M11**	Mozambique	-	-	*Rhipicephalus sp.*
MOC 3073	**M12**	Mozambique	-	-	*Rhipicephalus sp.*
RAS 3061	**SA1**	South Africa	M	-	*Rhipicephalus sp.*
RAS 3062	**SA2**	South Africa	M	Lion	*R. simus*
RAS 3070	**SA3**	South Africa	M	-	*Rhipicephalus sp.*
RAS 3074	**SA4**	South Africa	M	-	*Rhipicephalus sp.*
RAS 3075	**SA5**	South Africa	M	Blesbok	*R. evertsi evertsi*
RAS 3076	**SA6**	South Africa	F	Blesbok	*R. evertsi evertsi*
ST 3051	**S1**	St Tome	F	-	*R. decoloratus*
CV 3050	**C5**	Cape Verde	M	-	*Rhipicephalus sp.*
CV 3092	**C9**	Cape Verde	F	Vegetation	*R. sanguineus group*
Zbw 3063	**Z1**	Zimbwae	F	-	*Amblyomma sp.*
Zbw 3064	**Z2**	Zimbwae	F	-	*Amblyomma sp.*
Ang 3011	**A3**	Angola	-	Dog	*R. simus group*
Ang 3016	**A8**	Angola	-	Dog	*R. sanguineus group*
Ang 3017	**A9**	Angola	-	Dog	*R. sanguineus group*
Ang 3018	**A10**	Angola	F	-	*Rhipicephalus sp.*
Ang 3019	**A11**	Angola	M	-	*Rhipicephalus sp.*
Ang 3020	**A12**	Angola	-	Dog	*Rhipicephalus sp.*
Ang 3045	**A15**	Angola	M	Dog	*R. sanguienus group*
Ang 3046	**A16**	Angola	M	-	*Rhipicephalus sp.*
Ang 3047	**A17**	Angola	M	-	*Rhipicephalus sp.*
IT 3081	**IT2**	Italia	F	-	*R. turanicus*

3.3 Molecular studies

DNA from all samples were extracted using a commercial kit following manufactures instructions. The kit used was E.Z.N.A.® Tissue DNA Kit from Omega company. After DNA extraction, the quality and the level of contamination was analysed by a NanoDrop™ Spectrophotometer. The CR 1536, 1543, 1551, 1563 and Ang 3010, 3011, 3016, 3017, 3018, 3019, 3020, 3021, 3022 DNA samples were extracted for previous studies, specifically Coimbra-Dores (2014) and Coimbra-Dores *et al* (2017, 2016).

Mitochondrial markers for PCR reactions, namely COI mtDNA (Chitimia et al., 2010; Erster et al., 2013; Folmer et al., 1994), 16S rDNA (Black and Piesman, 1994) and 12S rDNA (Beati and Keirans, 2001) were chosen in order to obtain a molecular identification of our samples.

All primer sequences can be consulted on Table 2. PCR conditions used for each marker are provided in Appendix 9.1

An additional pair of primers were used in order to test the presence of pathogenic agents in some ticks collected from ill hosts, more specifically the EC16S (Cardoso et al., 2016) for Anaplasmatacea family of bacteria in a small sample group, the results are showed in Appendix 9.3.

Table 2: Primer sequences used for COI, 12S and 16S genes amplification

Marker	Foward	Reverse	Base pairs	Reference
COI	5'-GGTCAACAAATCATAAAGATATTGG-3'	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	700 bp	Folmer et al 1994
Cox1	5'-GGAACAATATATTTAATTTTTGG-3'	5'-ATCTATCCCTACTGTAAATATATG-3'	600 bp	Chitimia et al 2010
CoxT	5'-CCGCGATGAATATACTCTACTAAY-3'	5'-CCAGGATTTGGAATAATTTCTCAAA-3'	500 bp	Erster et al 2013
12S	5'-AAACTAGGATTAGATACCCT-3'	5'-AATGAGAGCGACGGGCGATGT-3'	400 bp	Beati and Keirans 2001
16S	5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3'	5'-TTACGCTGTTATCCCTAGAG-3'	300 bp	Black and Piesman 1994
16S_A	5'-TTGGGCAAGAAGACCCTATGAA-3'	5'-CCGGTCTGAACTCAGATCAAGT-3'	300 bp	Black and Piesman 1994

The PCR results were checked in a 2% agarose gel electrophoresis. After amplification, the PCR products correspondent to bands of interest were cleaned using the SureClean commercial kit by Bioline and send to STABVIDA for Sanger sequencing.

3.4 Data analysis

All the sequences obtained were treated in Sequencher v 4.0.5 (Sequencher, 1991), BioEdit v 7.2.5 (Tom Hall, 1997) and MAFFT v 7.222 (Katoh, 2013) . The phylogenetic analyses were performed in MEGA v 7 (Kumar et al., 2015) and RaXML v 8 (Stamatakis, 2014) Softwares. The outputs were visualized in Fig Tree v 1.4.3 (Rambaut, 2007).

For COI mtDNA and 12S rDNA, Maximum Likelihood (ML) method was performed with the application of the GRTGAMMA model. In the 16S rDNA case, Neighbour-Joining (NJ) method was applied, with the use of the p-distance model. Both analysis use bootstrap values obtained with 1000 replicates.

These analysis count not only with sequences generated in this study, but also with several *Rhipicephalus* species representative sequences acquired on GenBank. All the accession numbers, as well as correspondent laboratory codes are available on Appendix 9.2. Each analysis includes an out-group represented by two specimens either of genus *Hyalomma* or *Amblyomma genus*.

4. Results

4.1 Morphological results

Morphological results can be consulted in Table 1. Samples from Caldas da Rainha, São Facundo and Samora Correia were identified as *R. turanicus*, the remaining collected in Portugal were identified as *R. sanguineus s.l.*

From African samples, we had listed two *Amblyomma* sp., one *R. kochi*, one *R. decoloratus*, two *R. evertsi evertsi* and three *R. simus* and the all the others were *R. sanguineus s.l.* Two samples (A3 and C9) were identify until genus level, or even to group of species level. The Italian sample was classified as *R. turanicus.*

4.2 Molecular analysis

Out of our 66 samples collection, 56% were amplified with COI mtDNA, 59% with 12S rDNA, and 56% with 16S rDNA marker (Table 3). We obtained a total of 113 amplicons, from which 80 sequences were effectively sequenced. Of these, 76 sequences had enough quality to be used for phylogenetic analysis.

Portugal collected samples had a higher percentage of amplification (COI mtDNA and 16S rDNA= 78%; 12S rDNA= 76%) compared to Africa collected ones (COI mtDNA=29%; 16S rDNA=36%; 12S rDNA=32%).

Table 3: List of amplicons obtained in this study. (v) Represents the amplifications generated of each sample.

PORTUGAL					AFRICA				
Voucher	FCUL Lab Code	COI	12s	16S	Voucher	FCUL Lab Code	COI	12s	16S
SF 03003.1	SF1	v	v	v	GB 03006.1	G3		v	
SC 03005.1	P2	v	v	v	GB 03007.1	G4			
CR 1536	CR 5	v	v		MOC 003035	M1			
CR 1543	CR 8	v	v		MOC 003036	M2			
CR 1563	CR 10	v	v	v	MOC 003037	M3			
CR 1551	CR 13	v	v	v	MOC 3068	M10			
S 3089	ST1			v	MOC 3072	M11			
S 3097	ST3			v	MOC 3073	M12			
S 3100	ST4			v	RAS 3061	SA1			
S 3101	ST5				RAS 3062	SA2	v	v	v
S 3104	ST6			v	RAS 3070	SA3			
S 3105	ST7		v	v	RAS 3074	SA4			
S 3106	ST8			v	RAS 3075	SA5	v	v	v
Maf 003082	MF1	v	v		RAS 3076	SA6	v	v	v
Maf 003083	MF2	v	v		ST 3051	S1	v	v	
PV/CE1/14	CE1	v	v	v	CV 3050	C5		v	v
PG/CE2/11	CE2	v			CV3092	C9			v
PV/CE3/10	CE3	v	v		Zbw 3063	Z1	v		
PG/CE4/11	CE4	v	v		Zbw 3064	Z2	v		
PG/CE5/11	CE5	v	v	v	Ang 3010	A2	v	v	v
PG/CE7/11	CE7		v	v	Ang 3011	A3		v	v
PG/CE8/11	CE8	v	v	v	Ang 3016	A8			v
PG/CE9/11	CE9				Ang 3017	A9			v
PG/CE10/11	CE10	v	v	v	Ang 3018	A10			
PG/CE11/13	CE11	v	v	v	Ang 3019	A11			
PG/CE12/13	CE12	v	v	v	Ang 3020	A12			
PG/CE13/13	CE13	v	v	v	Ang 3021	A13	v	v	v
PF/RI3/16	RI3	v	v	v	Ang 3022	A14	v	v	v
PF/RI4/16	RI4	v	v	v	Ang 3045	A15	v	v	v
PF/RI5/16	RI5	v	v	v	Ang 3046	A16		v	
PF/RI6/16	RI6	v	v	v	Ang 3047	A17	v	v	
PF/RI7/16	RI7	v	v	v	ITALIA				
PF/RI9/16	RI9	v	v	v	Voucher	FCUL Lab Code	COI	12S	16S
PF/RI11/16	RI11	v	v	v	IT3081	IT2	v		
PF/RI10/16	RI10	v	v	v					
PF/RI12/16	RI12	v	v	v					
PF/RI17/16	RI17	v	v	v					

4.2.1 Sequence dataset analysis

All the descriptive information about the 352 obtained sequences is provided in Table 4.

Table 4: Sequence datasets information's. (m) total number of sequences in the data set, (n) total nucleotides present on each sequence, (C) marker conservative sites, (V) marker variable sites, (Pi) markers Parsimony-informative sites.

Sequence dataset	m	n	C	V	Pi
COI mtDNA	137	415	221	194	170
16S rDNA	101	156	72	84	77
12S rDNA	114	262	144	113	104

4.3 Phylogenetic analysis

We were able to identify two *Amblyomma hebraeum* (Koch, 1844), one *Rhipicephalus turanicus,* one *R. pusillus,* two *R. evertsi evertsi,* one *R. simus* and 28 *R. sanguineus* (Figs.4, 5 and 6). The samples from Angola (A3) and Cape Verde (C9) were not possible to identify until the species level with our available dataset, thus they were just identified until its' species group.

Amblyomma hebraeum was used as an out-group for the COI mtDNA phylogenetic analysis.

COI mtDNA and 16S rDNA provide similar topological structures with relatively high bootstrap values in the main branches (Figs. 4 and 5). The 12S rDNA tree allow to clarify some data within the species groups (Fig. 6). It was possible to distinguish two distinct clades of *R. sanguineus* lineages with the three markers. All the out-groups were highly supported in all of the trees Bootstrap values (BS) =88-100.

In COI mtDNA tree we were able to separate successfully the following species (BS=88-100): *R. sanguineus* in the tropical and temperate lineages, *R. leporis, R. guilhoni, R. turanicus* from CEM and EM lineages, *R. rossicus, R. pumilio, R. pusillus, R. microplus, R. annulatus, R. geigyi, R. evertsi evertsi, R. evertsi mimeticus, R. bursa, R. pravus, R. pulchellus, R. maculatus, R. muhsamae, R. simus, R. compositus, R. appendiculatus* and *R. zambeziensis.* The *R. sulcatus* specimen is poorly supported (BS=36) maybe because this is the only single-sequence in our dataset.

Still, in the COI mtDNA tree is possible to identify a sister species of *R. sanguineus* tropical lineage, the *R. guilhoni* (BS=97). *Rhipicephalus pumilio* and *R. rossicus* are also sister species (BS=98).

Some other close related groups showed good bootstrap values, like the *Boophilus* subgenera compared with the samples from *R. evertsi group* and *R. pravus* group (BS=85). Group *R. simus* compared with group *R. capensis* (BS=97).

For all the remaining groups the bootstrap values don't give us a secure information about the existent relations (BS≤85).

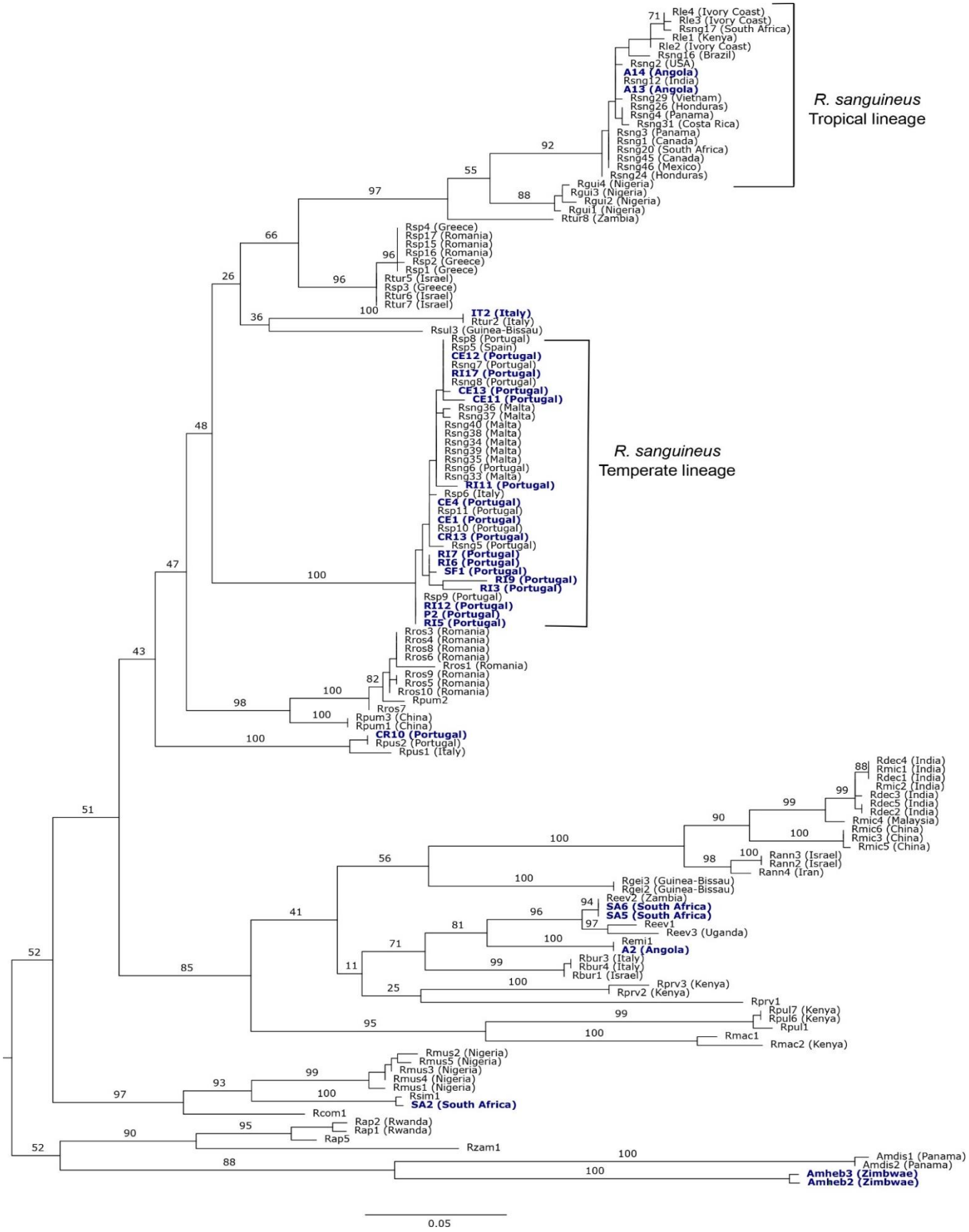

Figure 4: Maximum Likelihood phylogenetic tree of *Rhipicephalus* spp. based on COI mtDNA gene. Bootstrap values were obtained with 1000 replicates. In ( ) are the origin countries for each sequences. In case that the specimen origin was not available, they not figure in the tree. Blue bold letters show the sequences generated in this study.

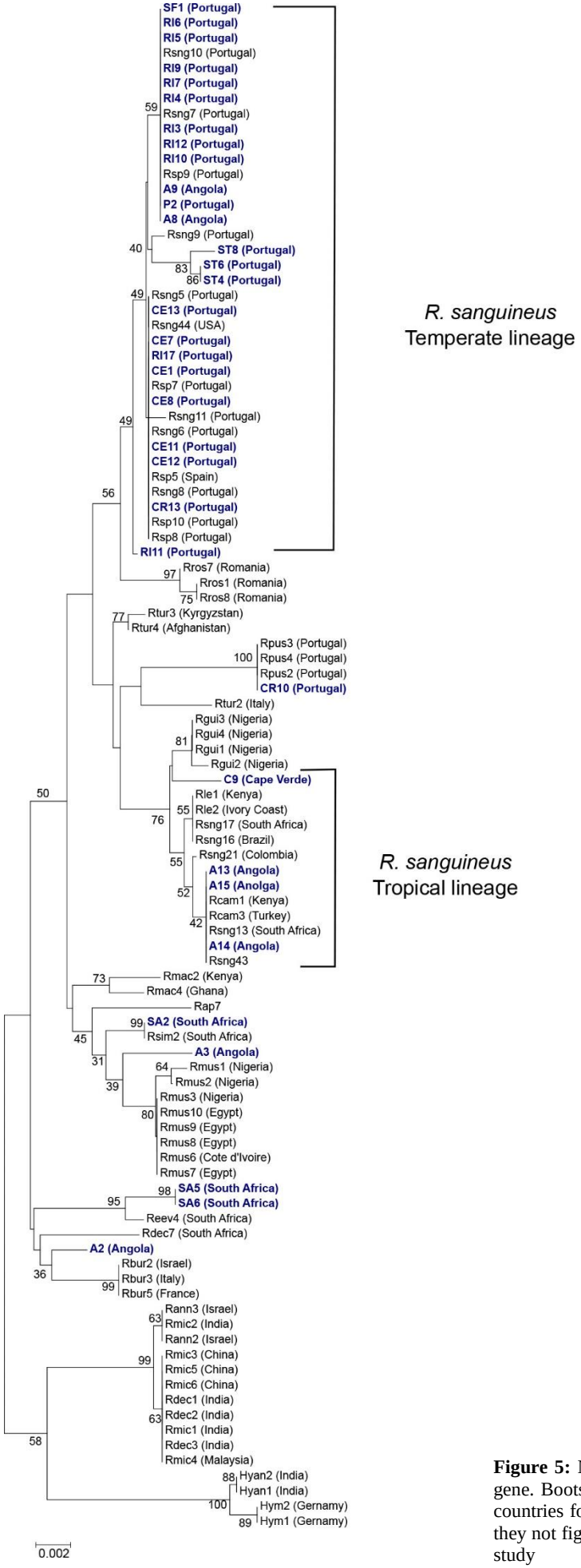

Figure 5: Neighbour-Joining tree of *Rhipicephalus* spp. based on 16S mtDNA gene. Bootstrap values were obtained with 1000 replicates. In ( ) are the origin countries for each sequence. In case that the specimen origin was not available, they not figure in the tree. Blue bold letters show the sequences generated in this study

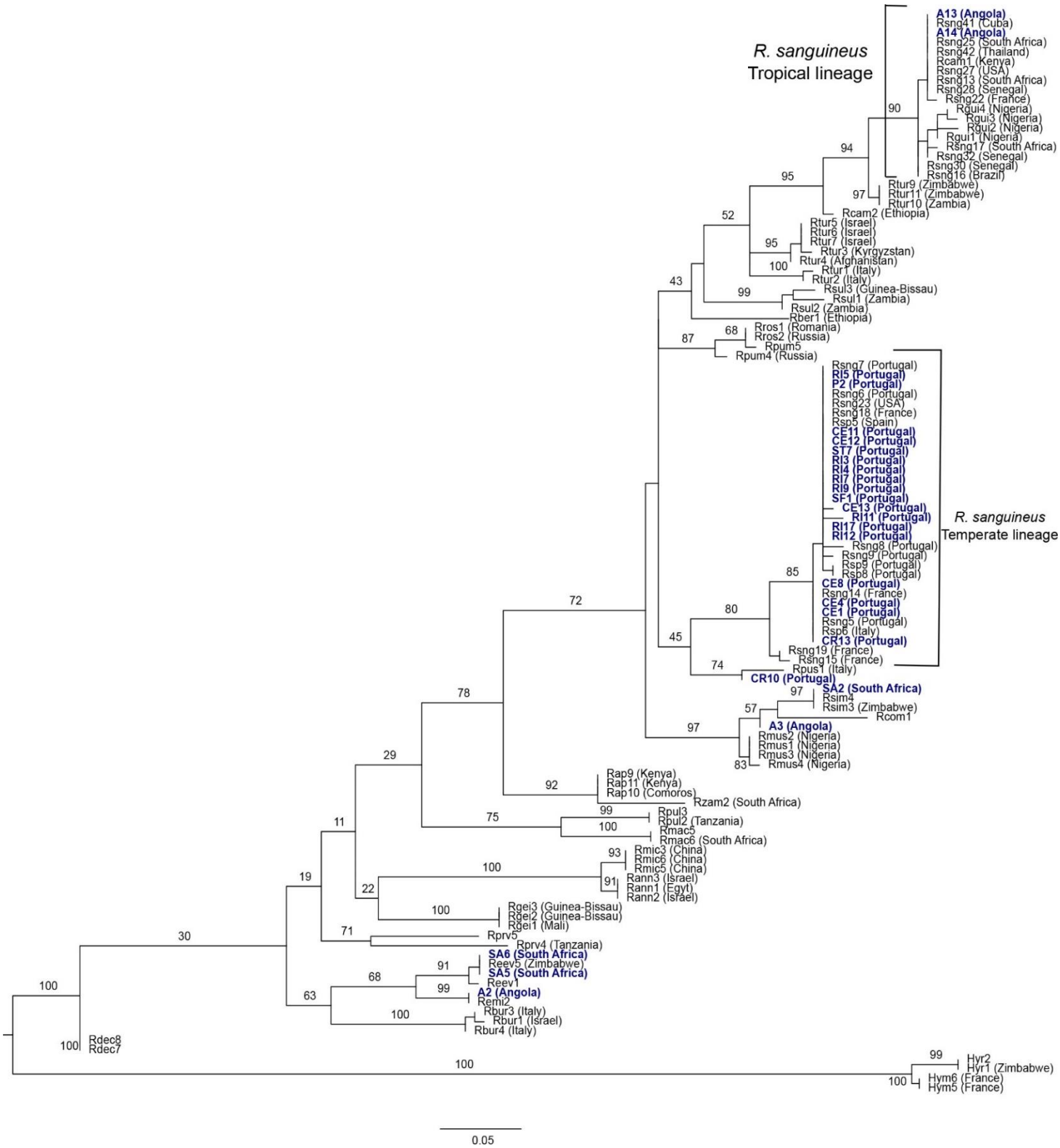

Figure 6: Maximum Likelihood phylogenetic tree of *Rhipicephalus* spp. based on 12S mtDNA gene. Bootstrap values were obtained with 1000 replicates. In ( ) are the origin countries for each sequence. In case that the specimen origin was not available, they not figure in the tree. Blue bold letters show the sequences generated in this study

12S rDNA marker dataset analyses allow us to separate with support the following species and subspecies (BS=85-100): *R. turanicus, R. sulcatus, R. sanguineus* from temperate lineage, *R. simus, R. muhsamae, R. appendiculatus, R. pulchellus, R. maculatus, R. microplus, R. annulatus, R. geigyi, R. evertsi evertsi, R. evertsi mimeticus, R. bursa* and *R. decoloratus.*

It was also possible to establish some relations between some species, like *R. sanguineus* tropical lineage that is closely related to the *R. turanicus* SeA lineage (BS=94), the sister species *R. pumilio* and *R. rossicus* (BS=87), and also the closely-related *R. simus* group and the *R. capensis* group (BS=97). The other bootstrap values do not make possible to assure any other relations.

With the 16S rDNA tree we separate with confidence (BS=95-99): *R. rossicus, R. pusillus, R. simus, R. evertsi evertsi* and *R. bursa*. This tree has some BS values between 70-80, but they are still not sufficient to establish any secure relations. That is the case of *R. maculatus* (BS=73), *R. turanicus* EM lineage (BS=77) and *R. muhsamae* (BS=80). *R. sanguineus* temperate and tropical lineages were also poorly supported, with BS=59 and 55 respectively. The remaining BS values do not assure us any secure evolutionary relationship.

All the trees BS values, distinguishing each group and his species can be consulted in Table 5.

Table 5: Bootstrap values distinguishing the group species and the species within them. (-) specie not supported by the tree, (x) specie not present on the tree.

	Bootstrap values (BS)		
	COI	**16S**	**12S**
***Boophilus* subgenera**	56	99	22
R. decoloratus	99	63	-
R. microplus	100	63	93
R. annulatus	98	-	91
R. geigyi	100	-	100
***R. evertsi* group**	71	19	63
R. evertsi evertsi	96	95	91
R. evertsi mimeticus	100	36	99
R. bursa	99	99	100
***R. pravus* group**	25	-	71
R. pravus	100	-	71
***R. simus* group**	93	31	97
R. simus	100	99	97
R. muhsamae	99	80	83
***R. capensis* group**	97	-	x
R. compositus	97	-	x
***R. appendiculatus* group**	90	45	92
R. appendiculatus	95	45	77
R. zambeziensis	x	-	99
***R. pulchellus* group**	95	73	75
R. pulchellus	99	-	99
R. maculatus	100	73	100
R. sanguineus group	43	24	12
Tropical lineage	92	55	90
Temperate lineage	100	56	80
R. turanicus EM	30	77	95
R. turanicus CEM	100	-	100
R. turanicus Sea	-	-	97
R. leporis	43	55	-
R. guilhoni	88	81	24
R. pumilio	100	97	87
R. pusillus	100	100	74
R. sulcatus	36	-	99
R. bergeoni	-	-	43
R. camicasi	-	-	95

5. Discussion

5.1 Molecular markers efficacy

The samples collected in Portugal presented a higher percentage of amplicons when compared with the African collected ones. This might due to the fact that the African collected samples are preserver for much longer, because we know that they were collected in the 80/90 decades. This "age" factor is also noticed when we compare the samples from CERVAS and RIAS, the first ones were collected with some years difference from the RIAS, and this last one also has originated more amplicons. Another factor to have in account is that African collected samples were not always preserved just in an ethanol solution. Some of them were conserved in solutions containing glycerol and/or formol and these types of compounds may interfere with the PCR reaction and also with DNA degradation. The same above reasons are pointed out to explain the fact that we were unable to amplify any sample from Mozambique.

The number of 16S rDNA amplifications obtained appears to have been more successful in our study, probably because the various possible primers combinations with this marker. The same results was expected for COI mtDNA, to which we dispose of three primers in order to distinguish more species, but the Folmer (1994) primers suggest to be the most effective.

5.2 Phylogenetic results

5.2.1 Markers tree evaluation

The different trees (COI mtDNA, 16S rDNA and 12S rDNA) obtained in this study show us different type of information's and they all complement each other. Low BS values on basal branches in our trees suggest that some species are missing which do not allow establishing secure evolutionary relationships. Or that the used markers are not the more informative ones to resolve our phylogeny. In fact, some group of species were not included in our dataset, because there is no data available. Another fact to consider is the number of sequences used to represent each species, depending on how they were available on GenBank. Despite these, our objectives were not compromise.

With our dataset we were not able to identify two samples, one from Angola, A3, and one from Cape Verde, C9. Nevertheless, we can suggest that the one from Angola belongs to *R. simus* group, the specimen from Cape Verde stands between *R. guilhoni* and *R. leporis* (Fig.4). This happened maybe because the markers used were not the ideal due to some mutations accumulation by the species. This could be resolved by design a new pair of primers basing the design on a close morphological related species or by optimization of PCR temperature of annealing. Although they are not secure named the results make sense if we have in account the origin of the specimens, both of them stand on the tree close to species that are reported in their areas.

COI mtDNA tree was the one that provided the most amount of information about relations inter species groups, once this marker is associated with species barcode it makes sense the quality of these results. The remaining used markers allowed a better clarification of the position of various species within the species groups, some of them less clarified by the COI based analysis.

Nevertheless, some relations within the tree still need to be clarified due to be low supported in ours phylogenies.

First, in COI mtDNA tree (Fig.4), *R. pravus* group was divided in two branches. Both Rprv2 and Rprv3, from Kenya, stand together, but Rprv1 clustered in a distant branch, in spite of a low BS value supports this division. This isolated sample was obtained by Murell *et al.* (2001) with an unknown origin. However, our results suggest that this specimen has already diverged from the others at a considerable time ago. In addition, on 12S rDNA tree (Fig.6) this separation is well established and corroborate the previous observation.

Another topic of interest is *R. decoloratus* and *R. microplus* groups. In the 16S rDNA tree, *R. decoloratus* group appears within the *R. evertsi* group. The two species sequences are mixed with each other, suggesting that they are not monophyletic groups. Probably, due to gene flow between these populations, or due to the occurrence of a past event of hybridization, the 16S rDNA gene of both morphologically isolated species had suffered an introgression, making this gene a bad marker to separate these species. In the COI mtDNA tree, there are two sequences of *R. microplus* (Rmic1 and Rmic2) that are in the major cluster of *R. decoloratus,* suggesting a possible misidentification. In order to clarify these unrevealed relationships, more studies are required, using other molecular markers or even trying new approaches, as the analyse of the whole genome.

Another species that seems not to be monophyletic is *R. camicasi*. In 16S rDNA tree, two sequences that were identified on GenBank as *R. camicasi,* Rcam1 and Rcam3, stand on our tropical lineage of *R. sanguineus.* This result can also suggest a misidentification. In 12S rDNA tree we have just one sequence of this species, Rcam2 obtained by Santos-Silva and Beati (2008, Unpublished) which appears isolated on the tree. As it was the first molecular identification based on the morphological evaluation of *R. camicasi* available on the literature, we considered it as the real *R. camicasi.* The Rcam1 sequence was obtained from GenBank deposited by Estrada-Peña (2016, Unpublished) who is currently working on the re-description of *R. camicasi.* However, this sequence clustered together with *R. sanguineus* tropical linage. The same happened with a sequence from Turkey (Hekimoğlu et al., 2016), Rcam3, that also appears within the *R. sanguineus* tropical lineage, and that was also identified by Estrada-Pena.

R. compositus has just two sequences on GenBank, and it is the only representing *R. capensis* group in this study. In our 12S rDNA analyses, Rcom1 appears within the *R. simus* group, suggesting that they share similar sequences on this mitochondrial region, although the BS value doesn´t support this inclusion. On the other hand, COI mtDNA tree was able to separate with support *R. compositus* from the *R. simus* group.

Other group that generates confusion is *R. pumilio* and *R. rossicus*. In COI mtDNA based tree, *R. rossicus* appear clustered with *R. pumilio* (Fig.4). *R. rossicus* was initially described as a sub-species of *R. sanguineus* (*R. sanguineus rossicus*) that latter started to be treated as a single species (Mihalca et al., 2015), and is currently on debate. Several authors try to repose the species status to *R. rossicus* mainly in Russia and Romania (Mihalca et al., 2015), which they defended to be a neglected species. It is possible, due to the referred similar morphology, that some specimens were misidentified with *R. sanguineus s.l.* (Dumitrache et al., 2014; Mihalca et al., 2015).

R. pumilio and *R. rossicus* molecular analyses with the nuclear marker ITS-2 suggests that both species are conspecific. Latrofa *et al.* (2014) pointed that *R. pumilio* and *R. rossicus* are 100% homologous when identified once again with ITS-2 marker. By the contrary,12S rDNA marker supports that *R. rossicus* is a single species (Dumitrache et al., 2014; Mihalca et al., 2015). However, the trees showed by Mihalca *et al*. (2015) when trying to analyse *R. rossicus*, shows that one of their sequences clustered together with *R. pumilio.* Unfortunately, the authors don´t show all the BS involved on species separation, then no further conclusion taken could be supported. Nuclear markers provide a much more reliable information to identify until genus level, therefore they closely relate these two species. Whereas the mitochondrial markers tend to better on species-specific identifications (Dantas-Torres et al., 2013; Latrofa et al., 2013; Lv et al., 2013).

Overall, previous studies support that both species are conspecific (Mihalca et al., 2015) are corroborated with our results, in which *R. rossicus* 12S rDNA and COI sequences clustered with high support with *R. pumilio* identified by Murrell *et al.* (2000) (BS=87 for 12S, and BS=100 for COI Maximum Likelihood trees).

R. evertsi group is considered to include two subspecies, *R. evertsi evertsi* and *R. eversti mimeticus* (Guglielmone and Nava, 2014). Due to the fact that a low number of sequences are available on GenBank, the addition of new sequences allowed us to separate these two species with high statistical support (BS=95-96) in 16S rDNA and COI based trees. In regard to *R. evertsi mimeticus* in 16S rDNA tree, our sequence appears alone, probably due to the fact that there is the single sequence available.

Our results suggest that maybe the subspecies *R. evertsi evertsi* and *R. evertsi mimeticus* should be considered two separated species, *R. evertsi* and *R. mimeticus*, what is supported by their clear morphological differentiation.

The species *R. guilhoni* and *R. leporis* are a set of tropical species that have not yet fully clarified. In our analysis both species cluster together with *R. sanguineus* tropical lineage as previous described in the literature (Dantas-Torres et al., 2013; Latrofa et al., 2013). *R. guilhoni* is isolated with moderate support in one clade in both 16S (BS=81) and COI (BS=88) phylogenies. Despite being included without support in the 12S tree within *R. sanguineus* tropical lineage, our results supports its status as a species. Its close relation to the referred lineage lead it to be considered as a sister species, although our phylogeny didn't solved this relation (BS 16S tree=76; BS COI tree=55).

R. leporis, although it is referred as morphologically differentiated from *R. sanguineus*-like morphologies (Hornok et al., 2017), it clustered within *R. sanguineus* tropical lineage in 16S and COI trees. However, in the COI phylogeny, some of its sequences grouped alone with some support (BS=71), what could support a very recent divergence between these clades. Nevertheless, more studies including other markers will be necessary to clarify *R. leporis* status as a single species.

R. turanicus identified specimen by Dantas-Torres (IT2), collected in Italy, in our analyses paired up with another *R. turanicus* (Rtur2) from Italy also identified by Dantas-Torres *et al.* (2013). In that same work, the authors explain that they have access to the type specimens identified by Fillipova (1997), and also they point up that species from Italy and Greece seems to be equivalent to that morphology-type.

In this study we have also obtained different clusters of *R. turanicus s.l.*, and we named the clusters based on their origin. This nomenclature is also been established in Coimbra-Dores *et al.* (2017, Submitted).

The first cluster includes the sample given by Dantas-Torres (IT2) and the Rtur2 (Dantas-Torres et al., 2013), both from Italy, and so we will being referring this cluster as "*R. turanicus* of Central East Mediterranean (CEM)" now on.

The second cluster grouped sequences from Afghanistan, Israel and Kyrgyzstan, so it will be referred as "East Mediterranean (EM)" group.

The last cluster include sequences from Zambia and Zimbabwe (Africa), so it was named "Southern-east African (SeA)".

We also identified a fourth cluster within our *R. turanicus* species in our COI mtDNA tree. The sequences belonging to the cluster were deposited on GenBank as *Rhipicephalus* sp. These sequences (Rsp1, Rsp2, Rsp4, Rsp15, Rsp16 and Rsp17) were originated from Greece and Romania, and so we called the cluster CEM II. These sequences were separated with a good support (BS=96) from remain clusters

The presence of all these different clusters, separated with high BS values, suggest that more than one species exists under *R. turanicus* designation.

5.2.2 *Boophilus* subgenera and *R. sanguineus s.l.* group separation

COI mtDNA tree strongly separate the *Boophilus* subgenera from the *R. sanguineus* complex of species, which have already proved to be paraphyletic groups (Burger et al., 2014; Murrell et al., 2001). *Boophilus* used to be a genus due to its highly different morphological and biological characteristics. However, based on molecular and morphological analyses, the genus *Boophilus* was transferred for the genus *Rhipicephalus* due to its clear inclusion in the group (Murrell and Barker, 2003). Nevertheless, the ecology aspects of these two groups reinforce their separation, since species belonging to *Boophilus* subgenera are one host ticks (Burger et al., 2014) and *R. sanguineus* group species use three hosts on their life cycle (Gray et al., 2013). The host preference seems also to be differentiated, since. *R. sanguineus* group use mainly domestic animals as hosts, especially dogs, and *Boophilus* species are more commonly found in cattle and in small wild animals (Dantas-Torres et al., 2017; Gray et al., 2013; Otranto et al., 2015a).

Moreover, *Boophilus* subgenera species are the major concern to livestock productions around the world, and there are no records of these ticks in another host group (Burger et al., 2014).

On 12S rDNA and 16S rDNA trees the results corroborate that previous *Boophilus* species belong within genus *Rhipicephalus*.

5.2.3 *R. sanguineus s.l.* group of species

For all the analysis done in this study, it was considered that *R. sanguineus s.l* includes the species from the temperate and tropical lineages. In regard of *R. turanicus s.l* it includes the species from the CEM, EM and SeA lineages.

R. sanguineus complex clusters obtained in all trees seems to corroborate some of these complex species morphological identifications, as the case of *R. pusillus*, *R. pumilio*, *R. bergeoni*, *R. sulcatus*, and *R. guilhoni*. Concerning *R. sanguineus* and *R. turanicus* lineages, more than one clade were associated to them.

In regard to *R. sanguineus s.l.*, all of our trees show two distinct clades, supporting what is suggested in the literature about the existence of two *R. sanguineus* lineages, the temperate and the tropical (Almeida et al., 2017; Dantas-Torres et al., 2013; Hornok et al., 2017; Jones et al., 2017; Zemtsova et al., 2016). The marker which better separated both lineages was COI mtDNA (temperate lineage BS=100, tropical lineage BS=92). Suggesting once again that there are two species under the *R. sanguineus* name.

All the specimens collected in the Portuguese districts stands on temperate lineage which corroborate the data from previous studies (Almeida et al., 2017; Dantas-Torres et al., 2013; Hornok et al., 2017; Sanches et al., 2016).

Most African collected samples used in this study clustered within the tropical lineage, as expected. Still, two sequences (A8 and A9) used in 16S rDNA tree were identified as belonging to *R. sanguineus* temperate lineage, despite their African origin. This type of result including a swap of lineages as not previously reported, according the research done in this study, therefore we may propose an explanation for this fact.

R. sanguineus s.l. main host are dogs, but they also were reported on cattle and livestock. All these animals can change their geographic location during their life time, either by migration or commercial exchanges. Globalization made possible a quick and efficient mobility for people and goods. Parasites are capable to travel with their hosts, therefor, a geographic location change is possible for a small tick through anthropogenic action.

The hosts of the two sequences' specimens collected in Africa, and identified as belonging to the temperate lineage, were dogs, which easily could had travelled with their owners from the European continent to Angola. These ticks may also have had travel using human as transport, both on their cloths or luggage.

We think that these ticks could have been originated from a European country like Portugal, where the temperate lineage prevails, and somehow ended to be collected on a dog in Africa, due to the strict relations and common travels and enhance of goods maintained in a daily basis between the two countries.

One of the reasons that took us to study tick African specimens, was due to previous information that put tick origins in this continent. African lineages seem to be the "ancestral lineages" from where all ticks' diversity diverged. This African origin theory was firstly molecularly worked by Murrell *et al.* (2000, 2001) and it was based on phylogeographic and phylogenetic results. Indeed, our African samples were well separated from the European ones, suggesting that same divergence.

Now concerning the *R. turanicus s.l* it seems to occur a disparity between the morphological and the phylogenetic results

The samples collected in Caldas da Rainha were identified as resemble *R. turanicus*-morphology. We have been able to obtain sequences from two of them, one was identified as *R. sanguineus* temperate lineage and the last one was identified as *R. pusillus.*

The same happened with the samples from São Facundo and Samora Correia, they also been associated with a *R. turanicus* morphology, and they turn out to be *R. sanguineus* temperate lineage. This issue was already been detected by other authors (Dantas-Torres et al., 2017; Estrada-Peña et al., 2017; Santos-Silva et al., 2011).

Despite the morphological resemble, all the samples clustered on the *R. sanguineus* temperate lineage, suggesting that within this lineage we are able to observe a phenotypic plasticity, as already been suggested by Coimbra-Dores *et al.* (2016). The *R. turanicus*-morphology is indeed given to the Mediterranean area (Coimbra-Dores 2016), but until today, there are no genetic records of them given to the Iberian Peninsula (Dantas-Torres *et al.* (2017). This corroborates the conclusion of a previous study by Santos-Silva *et al.* (2011) that point out that in their 15.000 ticks collected over the years, none of them were identified as *R. turanicus* in Western Iberian Peninsula. If we think in the Iberian Peninsula as a whole unit, the same misidentifications issues seem to be present in Spain too. There were authors that have used sequences classified as *R. turanicus* with Spanish origin that after all were identified as *R. sanguineus* (Hekimoğlu et al., 2016; Márquez et al., 2008). Despite the given area from the *R. turanicus* includes the Iberian Peninsula, this type of data suggest that the species is not genetically represented in this territory.

Our specimen identified as *R. pusillus* provide us some new insights too. This species is associated with poor information and also the only host associated to them is the European rabbit (*Oryctolagus cuniculus*) (Estrada-Peña et al., 2017; Gray et al., 2013). In a recent study Estrada-Peña (2017) point that exist records of this ticks parasitizing livestock and wild ruminants, but the authors believe that once again this might be a misidentification, and that the samples should be *R. bursa*. The host of our *R. pusillus* tick as a dog, being a domestic animal this information enters in contradiction with the remaining literature. But after all *R. pusillus* might not be strangers for domestic animals, because there are studies that put this species using cats and dogs as hosts (Otranto et al., 2017; Pennisi et al., 2015; Segura et al., 2014). In our country Rosa *et al.* (2013) and Coimbra-Dores (2014; 2016) had already identified *R. pusillus* parasitizing dogs. Another interesting data was given by Santos-Silva *et al.* (2011), when the authors suggest that in our country are reports of this species biting humans and wild carnivores, another hosts not typically associated to *R. pusillus.* These types of conflicts suggest that a new evaluation of this tick ecology is needed, especially regard species host choice.

5.2.4 *Rhipicephalus sanguineus* temperate lineage subclades

Despite most specimens appeared within their expected lineage, there were some interesting points that need to be more clarified. For example, it is possible to identify some non-supported subclades formation within the *R. sanguineus* temperate lineage. These subclades seem to suggest the existence of small populations sharing similar haplotypes, which appear to be diverging. At the first sight, these subclades seem to cluster by the collecting site, maybe related to an environmental niche.

In COI mtDNA tree, six different subclades are suggested within our 34 sequences dataset, as can be seen in Table 6. In 16S rDNA and 12S rDNA trees, three subclades are suggested, within the 37 and 32 sequences respectively, although they seem to be less informative.

Regarding the subclade I of the COI dataset (Table 6), it seems to include sequences collected in both Portugal and Spain, suggesting an Iberian Peninsula distribution. The COI tree subclade II include only Malta collected ticks, origin shared by almost all sequences of its sister subclade III. The exception in this latter group is the existence of one sequence collected in Portugal, what is suggestive of some tick-dispersion to new regions. However, we do not have access to sufficient information to confirm this theory. In the subclade IV clustered tick sequences collected in the central and northern Portugal, and in the V central and southern Portugal collected ticks. This could be a suggestion of a sympatric region located in the central region of Portugal, where several habitats characteristic of both north and southern Iberian Peninsula can be found. By last, the subclade VI includes only sequences obtained from ticks collected in the southern Portugal, suggesting the existence of more than one possible population in the region.

With respect to the 12S and 16S based trees, both present only three subclades, showing less variability than the COI region. All subclades share Portugal as an origin region, although the 16S subclade III and the 12S subclade I also include ticks collected in the USA, showing to be more geographically distributed. In addition, two samples of the 16S subclade I were collected in Angola, suggesting a possible invasion of this African country through anthropic action, and both 12S subclades II and III include sequences of ticks collected in France.

Moreover, none of these clades formation seems to be correlated with a specific host-group.

Taking these results into account, the geographic origin appears to have some correlation with these subclades formation. Nevertheless, most species where collected in the Iberian and Italic Peninsulas, south France and Malta, which share temperate climates. This could suggest that ticks-population environmental niches, even within a lineage, should be associated to small-scale ecosystems. The presence of specific mammals in the region, being them dogs, foxes, hedgehogs or others, do not seems to be a limitation to their dispersal.

Interestingly, the major difference between ours and GenBank' sequences are the hosts where the ticks were collected from. All the sequences used from GenBank were of ticks collected in dogs, while our sampling counts not only with dogs, but also, wildlife and livestock. This is strongly indicative of a biased sampling, since the sampling effort seems to be centred in the domestic animal that lives closer to us, the dog. It is easier to collect ticks from dogs than from hedgehogs, since the dog lives within humans and the hedgehogs are nocturne animals, and ticks are only collect from them when one is collected by a wildlife centre due to illness.

That been said, domestic animals, mainly dogs, continue to be considered the main host associate with these ticks. Even thinking that dogs have privileged contact with humans, they are not the most dangerous reservoir for TBD, especially because we tend to have our domestic animals free from parasites (Millán et al., 2016).

Table 6: Specimens information within each subclades obtained in the study

Subclades

COI mtDNA tree

Subclade I			
GenBank sequences	Host	Origin	
Rsng7	Dog (Canidae)	Santarém	Portugal
Rsng8	Dog (Canidae)	Santarém	
Rsp5	Dog (Canidae)	-	Spain
Rsp8	Dog (Canidae)	-	Portugal
Our sequences	Host	Origin	
RI17	Fox (Canidae)	Faro	Portugal
CE12	Hedgehog (Erinaceidae)	Guarda	
CE11*	Hedgehog (Erinaceidae)	Guarda	
CE13*	Hedgehog (Erinaceidae)	Guarda	

Subclade II		
GenBank sequences		Origin
Rsng36	Dog (Canidae)	Malta
Rsng37	Dog (Canidae)	

Subclade III			
GenBank sequences	Host	Origin	
Rsng6	Dog (Canidae)	Santarém	Portugal
Rsng33	Dog (Canidae)	-	Malta
Rsng34	Dog (Canidae)	-	Malta
Rsng35	Dog (Canidae)	-	Malta
Rsng38	Dog (Canidae)	-	Malta
Rsng39	Dog (Canidae)	-	Malta
Rsng40	Dog (Canidae)	-	Malta

Subclade IV			
GenBank sequences	Host	Origin	
Rsp10	Dog (Canidae)	-	Portugal
Rsp11	Dog (Canidae)	-	
Our sequences	Host	Origin	
CR13	Hedgehog (Erinaceidae)	C. da Rainha	Portugal
CE1	Fox (Canidae)	Viseu	
CE4	Fox (Canidae)	Guarda	

Subclade V			
Our sequences	Host	Origin	
SF1*	-	São Fecundo	Portugal
RI6	Fox (Canidae)	Faro	
RI7	Fox (Canidae)	Faro	
RI3*	Fox (Canidae)	Faro	
RI9*	Fox (Canidae)	Faro	

Subclade VI			
GenBank sequences	Host		Origin
Rsp9	Dog (Canidae)	-	Portugal
Our sequences	Host		Origin
P2	Dog (Canidae)	Samora Correia	Portugal
RI5	Fox (Canidae)	Faro	
RI12	Fox (Canidae)	Faro	

16S rDNA tree

Subclade I			
GenBank sequences	Host	Origin	
Rsp9	Dog (Canidae)	-	Portugal
Rsng7	Dog (Canidae)	Santarém	
Rsng10	Dog (Canidae)	Santarém	
Our sequences		Origin	
A8	-	-	Africa
A9	-	-	
SF1	-	São Fecundo	Portugal
P2	Dog (Canidae)	Samora Correia	
RI3	Fox (Canidae)	Faro	
RI4	Fox (Canidae)	Faro	
RI5	Fox (Canidae)	Faro	
RI6	Fox (Canidae)	Faro	
RI7	Fox (Canidae)	Faro	
RI9	Fox (Canidae)	Faro	
RI10	Fox (Canidae)	Faro	
RI12	Fox (Canidae)	Faro	

Subclade II			
GenBank sequences	Host	Origin	
Rsng9	Dog (Canidae)	Lisbon	Portugal
Our sequences	Host		Origin
ST4	Cow (Bovidae	Santarém	Portugal
ST6		Santarém	
ST8	-	Santarém	

Subclade III			
GenBank sequences	Host	Origin	
Rsng5	Dog (Canidae)	Santarem	Portugal
Rsng11*			
Rsng44		-	USA
Rsp7		-	Portugal
Rsng6	Dog (Canidae)	Santarem	Portugal
Rsng8			
Rsp5		-	Spain
Rsp8		-	Portugal
Rsp10	-	-	
Our sequences	Host	Origin	
CE1	Fox (Canidae)	Viseu	Portugal
CE7		Guarda	
CE8			
CE13		C. da Rainha	
CR13			
CE11		Guarda	
CE12			
CE13			

12S rDNA tree

Subclade I			
GenBank sequences	Host	Origin	
Rsng6	Dog (Canidae)	Santarém	Portugal
Rsng7			
Rsng18		-	France
Rsng23		Arizona	USA
Rsp5		-	Spain
Rsng8*		Lisbon	Portugal
Rsng9*			
Rsp8*		-	
Rsp9*		-	
Our sequences	Host	Origin	
SF1	-	São Fecundo	Portugal
P2	Dog (Canidae)	Samora Correia	
ST7	Sheep (Bovidae)	Santarém	
RI3	Fox (Canidae)	Faro	
RI4			
RI5			
RI7			
RI9			
CE11	Hedgehog (Erinaceidae)	Guarda	
CE12			
CE13*	Hedgehog (Erinaceidae)	Guarda	
RI11*	Fox (Canidae)	Faro	
RI12			
RI17	Hedgehog (Erinaceidae)		

Subclade II			
GenBank sequences	Host	Origin	
Rsp6	Dog (Canidae)	-	Italy
Rsng5		Santarem	Portugal
Rsng14		-	France
Our sequences	Host	Origin	
CE1	Fox (Canidae)	Viseu	Portugal
CE4			
CE8	Hedgehog (Erinaceidae)	Guarda	
CR13			

Subclade III			
GenBank sequences	Host	Origin	
Rsng 15	Dog (Canidae)	-	France
Rsng19		-	

* Specimen present some mutations in relation the the remaining elements of the suggested subclade

As already mentioned, foxes are the main wild-host where ticks' subclades where obtained in the present study. In our country a few studies were done in order to investigate the pathogenic agents that foxes might carry (Cardoso et al., 2015, 2013; Maia et al., 2014a). All the foxes from the studies were positive from some kind of pathogen, proving the importance of monitoring these species. Also on those previous works, ticks parasitizing foxes were identified as *R. sanguineus s.l.,* which is concordant with our results.

Hedgehogs are another species present on our subclades, and they are one of the principal natural reservoirs of Crimean-Congo haemorrhagic fever virus (CCHF) (Mihalca et al., 2015). We cannot ignore that CCHF is a re-emergent zoonosis that were already reported in the Iberian Peninsula (Palomar et al., 2017). Although the previous study does not mention hedgehogs, this proves once again that is essential to be aware of vectors expanding geographic distributions, and for that it is necessary that a good surveillance network is already implemented.

Co-evolution between hosts and parasites, is a notion which implies that within the parasitic relationship both hosts and parasites acquire new ways that allow them either to defend from parasite in case of the host, or the ability to infect in a more effective way, in the parasites case (Kim, 1985; Šimo et al., 2017).

Our sampling suggests that *R. sanguineus* temperate lineage ticks are not specialist parasites, but generalists. Nevertheless, further studies will be necessary to confirm this indication, since as referred before, most studies seem to be biased in terms of host-origin. A worldwide spread parasite with the ability to parasitize several different species will represent an increased challenge to control and prevention systems.

McCoy *et al.* (2013) suggested that ticks follow a pattern of being global generalists and local specialists. This observation corroborates what is observed in many studies, since they are worldwide distributed but they could be locally adapted to a reduced group of hosts (Morley et al., 2016).

Three factors must be taken in to account when trying to explore tick-host specificity: biological, phylogenetic and geographical specificities. The first one refers to intrinsic biological characteristics of ticks, such as the digestion of the different hosts' bloods. Second one points out the evolutionary relationships among the various hosts used for obtain blood meals, and the third factor contemplates the geographic distribution and habitat characteristics (Esser et al., 2016; Nava and Guglielmone, 2013). McCoy *et al.* (2013) refers that other variables could contemplate quantitative approaches of host specificity, such as counting the number of hosts used in a complete life cycle, or qualitative approaches that measure ticks' fitness on different hosts.

Despite our study doesn't have as major purpose to determine any host specificity, the obtained results did not suggested a tick-host specificity. These results are not concordant with some previous works, where this type of specificity was reported (Esser et al., 2016). In this some study the authors collected samples only from a Panama region, and on their sampling *R. sanguineus s.l.* was represented and prove to have some tendency for order Carnivora hosts. Nevertheless, the authors also highlighting relations between collected ticks and domestic animals (such as dogs, cats and cows), and once more *R. sanguineus s.l,* were associated with all of them.

Host choice and diversity are important to establish biosystematics relations, but they are not the only factors capable of inducing adaptation. Some authors defend that biogeographic abiotic factors and environmental stress have more influence in how ticks locally adapt (McCoy et al., 2013; Nava and Guglielmone, 2013). In our case, we don't have data that allow us to fully clarify this topic, but we can relate this with a common issue of current environmental stress: climatic changes. Andersen and Davis (2016) made a study in order to evaluate if climatic changes will affect tick ecology and epidemiology of TBDs.

These authors reported that, especially due to the temperature increments, ticks tend to expand their habitats further and, therefore, this will bring new species into new territories where they had not been reported before. Consequently, the TBD epidemiology will probably change as well, as new diseases might emerge/ re-emerge in the different regions of the globe.

All that our subclades tell us in regard of host choice is a suggestion of a preference until order, family level from our sampling of *R. sanguineus* temperate lineage. These results are concordant with some previous works made especially to evaluate host specificity of ticks, where this type of specificity was reported (Esser et al., 2016). In our sampling the hosts are mainling foxes and dogs, share the same order (Carnovira) and family (Canidae). The same happens with sheeps and cows, order Artiodactyla and family Bovidae, finally the hedgehog family, Erinaceida.

Due to zoonotic potential of TBDs, morphological, ecological and molecular studies should be made in order to alert institutions responsible for maintaining public health for tick as vectors of serious diseases, and for that a threat for human health.

6. Conclusions

Rhipicephalus ticks are main vectors of several TBD, and a clear taxonomic classification of these parasites is essential to not only identify associated diseases, but also to proceed with health impact studies and suggest Acari control methods.

Our study, based on *Rhipicephalus* species identification through the use of molecular markers, allowed us to respond to several area issues. First, our results suggest that the *R. sanguineus* type morphology found in Portuguese territory is mainly the *R. sanguineus* temperate lineage, and the same morphology found on Africa corresponds mainly to the tropical lineage. Moreover, the temperate lineage suggested to comprehend several populations that could be diverging, supporting the recent expansion observed within this group of species

The obtained phylogeny also evidenced that *R. eversti evertsi* and *R. evertsi mimeticus* should be considered separated entities, maybe even species on their own, based in the mitochondrial divergence found and in their morphological differences.

Moreover, although *R. turanicus*-type-morphology continues to be identified in the Iberian Peninsula, molecular results do not support the existence of any of its lineages in that geographical area.

In addition, no correlations could be taken relating *Rhipicephalus*-host specificity due to the biased sampling methods performed, but this is a problem observed through the generality of this genus literature.

It was also possible to establish that the three molecular markers used in this work were efficient to the identification of the different species included in the genus *Rhipicephalus*.

We believe that our objectives were achieved with success, and that our findings and data will be extremely useful for future studies relating these important vectors of disease.

7. Future perspectives

For further studies, it is proposed, based on our study results that is important to always combine morphologic and molecular data in order to be possible to achieve more reliable and replicable results. In addition, it is also very important to include in the field work a methodology where the ticks' hosts-associated information will be less biased, since the current studies are not allowing that further conclusions relating the host-specialization to be taken any further. Also, it is important to perform more studies relating wildlife and livestock groups of hosts, since this information is lacking.

Ticks dynamic and population studies are also relevant investigations to carry out, since that type of knowledge will provide key results for clarify issues brought out from phylogenetic and evolutionary studies.

Concluding, ticks and TBD still need to become a more exposed topic for the general population a since this issue will not only affect in terms of economy animal's owners and explorations in the future, as all society must be aware of (re-)emergent zoonosis associated with these parasites due to the distribution range alterations that they will experience due to global changes.

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9. Appendices

9.1 Molecular protocol

PCR Mix conditions

COI,Cox1,CoxT

	[Stock]	[Final]	Vsample (uL)
ddH2O	-	-	8,15
Buffer	10x	1x	2,5
dNTPs	2.5mM	0.1mM	1
MgCl2	50mM	2,5 mM	1,25
Primer F	10uM	0.4uM	1
Primer R	10uM	0.4uM	1
BSA	2ug/uL	0.16ug/uL	2
taq	5U/ul	0.5U	0,1
DNA	-	-	8
		V_{PCR}=	25

16S, 12S, EC_16S

	[Stock]	[Final]	Vsample (uL)
ddH2O	-	-	10.15
Buffer	10x	1x	2.5
dNTPs	2.5mM	0.1mM	1
MgCl2	50mM	2,5mM	1,25
Primer F	10uM	0.4uM	1
Primer R	10uM	0.4uM	1
taq	5U/ul	0.5U	0,1
DNA	-	-	8
		V_{PCR}=	25

Optimize termocycler conditions: SimpliAmp Thermal Cycler

- **16S +2_F e 16S -1_R**

95°	5min	
95°	1min	
56°/57°	45sec	] x40
72°	1min30sec	
72°	7min	

- **16S_F e 16S_R**

94°	2min	
94°	45sec	
40°	45sec	] x35
72°	45sec	
72°	7min	

- **COI, Cox1, CoxT**

94°	5min	
94°	1min	
45°/50°	1min	] x40
72°	1min30sec	
72°	7min	

- **12S (Touchdown PCR)**

94°	5min	
94°	45sec	
53°/45°	45 sec	] x35
72°	45 sec	
72°	7min	

- **EC_16S**

95°	5min	
95°	1min	
65°	1min	] x40
72°	1min30sec	
72°	7min	

9.2 Accession numbers of GenBank sequences used on phylogenetic studies

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Amblyomma hebraeum (Koch, 1844)	**Amheb2**	**Zimbwae**				**This study**
	Amheb3	**Zimbwae**				
Amblyomma dissimile (Koch, 1844)	Amdis1	Panama			KF200168.1	Miller et al 2013 (Unpublished)
	Amdis2	Panama			KF200170.1	
Hyalomma marginatum (Koch 1844)	Hym1	Gernamy		KU987256.1		Chtimia et al 2016
	Hym2	Gernamy		KY111454.1		
	Hym5	France	KX000613.1			Vial et al 2016
	Hym6	France	KX000629.1			
Hyalomma rufipes (Koch, 1844)	Hyr1	Zimbabwe	AF150033.1			Beati and Keirans 2001
	Hyr2		AF031856.1			Murrell et al 1999
Hyalomma anatolicum excavatum (Koch, 1844)	Hyan1	India		KP210047.1		Chhillar et al 2014 (Unpublished)
	Hyan2	India		KP210048.1		Chhillar et al 2014 (Unpublished)
Rhipicephalus pusillus (Gil Collado, 1938)	Rpus1	Italy	KC243815.1		KC243901.1	Dantas-Torres et al 2013
	Rpus2	Portugal		KU513961.1	KU556747.1	Almeida et al 2016
	Rpus3	Portugal		KU513962.1		
	Rpus4	Portugal		KU513963.1		
	CR10	**Portugal**				**This study**

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus zambeziensis (Walker, Norval & Corwin, 1981)	Rzam1				AY008683.1	Murrell et al 2001
	Rzam2	South Africa	DQ849238.1			Mtambo et al 2007
Rhipicephalus turanicus (Pomerantsev 1936)	Rtur1	Italy	AF483243.1			Bernasconi et al 2002
	Rtur2	Italy	KC243823.1	KC243863.1	KC243909.1	Dantas-Torres et al 2013
	Rtur3	Kyrgyzstan	KT382493.1	KT382459.1		Zemtsova et al 2016
	Rtur4	Afghanistan	KT382479.1	KT382445.1		Zemtsova et al 2016
	Rtur5	Israel	KF958362.1		KJ409972.1	Shenkar and Gottlieb 2013
	Rtur6	Israel	KF958361.1		KJ409970.1	Shenkar and Gottlieb 2013
	Rtur7	Israel	KF958363.1		KJ409971.1	Shenkar and Gottlieb 2013
	Rtur8	Zambia			DQ859260.1	Mtambo et al 2007
	Rtur9	Zimbabwe	AF150017.1			Beati and Keirans 2001
	Rtur10	Zambia	DQ849232.1			Mtambo et al 2007
	Rtur11	Zimbabwe	FJ536569.1			Santos-Silva and Beati 2008 (Unpublished)
	IT2	**Italy**				**This study**
Rhipicephalus microplus (Canestrini, 1888)	Rmic1	India		KP210054.1	KP792589.1	Chhillar et al 2015 (Unpublished)
	Rmic2	India		KP210053.1	KP792588.1	Chhillar et al 2015 (Unpublished)
	Rmic3	China	KF583590.1	JX051072.1	JX051129.1	Lv et al 2014
	Rmic4	Malaysia		KP210059.1	KM246867.1	Low et al 2015
	Rmic5	China	KF583585.1	JX051068.1	JX051125.1	Lv et al 2014
	Rmic6	China	KF583582.1	JX051062.1	JX051119.1	Lv et al 2014
Rhipicephalus annulatus (Say, 1821)	Rann1	Egyt	EU921773.1			Labruna et al 2008
	Rann2	Israel	KF219717.1	KF219727.1	KF219738.1	Erster et al 2013
	Rann3	Israel	KF219718.1	KF219728.1	KF219739.1	Erster et al 2013
	Rann4	Iran			JX422019.1	Nabian et al 2012

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus bursa (Canastrini & Fanzago, 1878)	Rbur1	Israel	KF219719.1		KF219740.1	Erster et al 2013
	Rbur2	Israel		KF219729.1		Erster et al 2013
	Rbur3	Italy	KC243833.1	KC243871.1	KC243927.1	Dantas-Torres et al 2013
	Rbur4	Italy	KC243834.1		KC243928.1	Dantas-Torres et al 2013
	Rbur5	France		KX553962.1		Grech-Angelini et al 2016
Rhipicephalus muhsamae (Morel & Vassiliades, 1965)	Rmus1	Nigeria	KC243829.1	KC243868.1	KC243922.1	Dantas-Torres et al 2013
	Rmus2	Nigeria	KC243830.1	KC243869.1	KC243923.1	Dantas-Torres et al 2013
	Rmus3	Nigeria	KC243831.1	KC243870.1	KC243924.1	Dantas-Torres et al 2013
	Rmus4	Nigeria	KC243832.1		KC243925.1	Dantas-Torres et al 2013
	Rmus5	Nigeria			KC243926.1	Dantas-Torres et al 2013
	Rmus6	Cote d'Ivoire		KY111471.1		Langguth et al 2017
	Rmus7	Egypt		KY111466.1		Langguth et al 2017
	Rmus8	Egypt		KY111459.1		Langguth et al 2017
	Rmus9	Egypt		KY111463.1		Langguth et al 2017
	Rmus10	Egypt		KY111467.1		Langguth et al 2017
Rhipicephalus guilhoni (Morel & Vassiliades, 1963)	Rgui1	Nigeria	KC243811.1	KC243851.1	KC243897.1	Dantas-Torres et al 2013
	Rgui2	Nigeria	KC243812.1	KC243852.1	KC243898.1	Dantas-Torres et al 2013
	Rgui3	Nigeria	KC243813.1	KC243853.1	KC243899.1	Dantas-Torres et al 2013
	Rgui4	Nigeria	KC243814.1	KC243854.1	KC243900.1	Dantas-Torres et al 2013
Rhipicephalus evertsi evertsi (Neumann, 1897)	Reev1		AF031861.1		AF132835.1	Murrell et al 2000
	Reev2	Zambia			DQ859259.1	Mtambo et al 2007
	Reev3	Uganda			AB934398.1	Nakayima et al 2014

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus evertsi evertsi (Neumann, 1897)	Reev4	South Africa		KJ613642.1		Halajian et al 2016
	Reev5	Zimbabwe	AF150052.1			Beati and Keirans 2001
	SA5	**South Africa**				**This study**
	SA6	**South Africa**				**This study**
Rhipicephalus evertsi mimeticus (Werder, 1992)	Remi1				AF132836.1	Murrell et al 2000
	Remi2		AF031862.1			Murrell et al 1999
	A2	**Angola**				**Coimbra-Dores et al 2017 (Unpublished)**
Rhipicephalus rossicus (Yakimov & Kol-Yakimova, 1911)	Rros1	Romania	KJ425484.1	KU848178.1	KU848179.1	Dumitrache et al 2014
	Rros2	Russia	AF150021.1			Beati and Keirans 2001
	Rros3	Romania			JX394213.1	Marosi et al 2012 (Unpublished)
	Rros4	Romania			JX394214.1	Marosi et al 2012 (Unpublished)
	Rros5	Romania			JX394215.1	Marosi et al 2012 (Unpublished)
	Rros6	Romania			JX394216.1	Marosi et al 2012 (Unpublished)
	Rros7	Romania		KX793732.1	KX757897.1	Hornok et al 2016
	Rros8	Romania		KX793733.1	KX757898.1	Hornok et al 2016
	Rros9	Romania			KX757899.1	Hornok et al 2016
	Rros10	Romania			KX757900.1	
Rhipicephalus camicasi (Morel, Mouchet & Rodhain, 1976)	Rcam1	Kenya	KU746974.1	KU746973.1		Estrada-Pena et al 2016 (Unpublished)
	Rcam2	Ethiopia	FJ536556.1			Santos-Silva and Beati 2008 (Unpublished)
	Rcam3	Turkey		KU664368.1		Hekimoglu et al 2016
Rhipicephalus sulcatus (Neumann, 1908)	Rsul1	Zambia	FJ536565.1			Santos-Silva & Beati 2008 (Unpublished)
	Rsul2	Zambia	FJ536564.1			Santos-Silva & Beati 2008 (Unpublished)
	Rsul3	Guinea-Bissau	KU568504.1		KU568514.1	Zuquete et al 2017

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus geigyi (Aeschlimann & Morel, 1965)	Rgei1	Mali	KF569939.1			McCoy et al 2014
	Rgei2	Guinea-Bissau	KU568501.1		KU568512.1	Zuquete et al 2017
	Rgei3	Guinea-Bissau	KU568502.1		KU568513.1	Zuquete et al 2017
Rhipicephalus compositus (Neumann, 1897)	Rcom1		AF031860.1		AF132834.1	Murrell et al 2000
Rhipicephalus simus (Koch, 1844)	Rsim1				AF132840.1	Murrell et al 2000
	Rsim2	South Africa		KJ613641.1		Halajian et al 2016
	Rsim3	Zimbabwe	AF150019.1			Beati & Keirans 2001
	Rsim4		AF031866.1			Murrell et al 2000
	SA2	**South Africa**				**This study**
Rhipicephalus pumilio (Schulze, 1935)	Rpum1	China			HM193877.1	Sun et al 2010 (Unpublished)
	Rpum2				AY008684.1	Murrell et al 2000
	Rpum3	China			HM193878.1	Sun et al 2010 (Unpublished)
	Rpum4	Russia	AF150023.1			Beati & Keirans 2001
	Rpum5		AY008690.1			Murrell et al 2000

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus leporis (Pomerantsev, 1946)	Rle1	Kenya		KX793743.1	KX757911.1	Hornok et al 2016
	Rle2	Ivory Coast		KX793744.1	KX757912.1	Hornok et al 2016
	Rle3	Ivory Coast			KX757913.1	Hornok et al 2016
	Rle4	Ivory Coast			KX757917.1	Hornok et al 2016
Rhipicephalus pulchellus (Gerstäcker, 1873)	Rpul1				AY008682.1	Murrel et al 2001
	Rpul2	Tanzania	AF150024.1			Beati and Keirans 2001
	Rpul3		AF031864.1			Murrel et al 2001
	Rpul6	Kenya			KR262490.1	Hawkins et al 2015
	Rpul7	Kenya			KR262487.1	Campana et al 2016
Rhipicephalus maculatus (Neumann, 1901)	Rmac1				AY008681.1	Murrel et al 2000
	Rmac2	Kenya		KP858499.1	KP862678.1	Mwamuye and Villinger 2015
	Rmac4	Ghana		KY413797.1		Chitimia et al (Unpublished)
	Rmac5		AY008687.1			Murrel et al 2001
	Rmac6	South Africa	AF150026.1			Beati and Keirans 2001
Rhipicephalus bergeoni (Morel & Balis, 1976)	Rber1	Ethiopia	KX377408.1			Kumsa et al 2016 (Unpublished)
*Rhipicephalus pravu*s (Dönitz, 1910)	Rprv1				AF132837.1	Murrell et al 2000
	Rprv2	Kenya			KT307494.1	Mwamuye et al 2015
	Rprv3	Kenya			KT956187.1	Campana et al 2016
	Rprv4	Tanzania	AF150025.1			Beati and Keirans 2001
	Rprv5		AF133055.1			Murrell et al 2000
Rhipicephalus appendiculatus (Neumann 1901)	Rap1	Rwanda			DQ901363.1	Mtambo et al 2006
	Rap2	Rwanda			DQ901362.1	Mtambo et al 2006
	Rap5				AF132833.1	Murrell et al 2000
	Rap7			L34301.1		Black and Piesman 1994
	Rap9	Kenya	DQ901320.1			Mtambo et al 2006
	Rap10	Comoros	DQ901317.1			Mtambo et al 2006
	Rap11	Kenya	DQ901316.1			Mtambo et al 2006

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus decoloratus (Koch, 1844)	Rdec1	India		KP210070.1	KP792569.1	Chhillar et al 2015 (Unpublished)
	Rdec2	India		KP210068.1	KP792575.1	Chhillar et al 2015 (Unpublished)
	Rdec3	India		KP210062.1	KP792571.1	Chhillar et al 2015 (Unpublished)
	Rdec4	India			KP792594.1	Chhillar et al 2015 (Unpublished)
	Rdec5	India			KP792576.1	Chhillar et al 2015 (Unpublished)
	Rdec7	South Africa	EU921774.1	EU918193.1		Labruna et al 2008 (Unpublished)
	Rdec8	Mali	KF569940.1			Mccoy et al 2014
Rhipicephalus sanguineus (Latreille,1806)	Rsng1	Canada			KX360403.1	Ondrejicka et al 2017
	Rsng2	Kentucky			KX360367.1	Ondrejicka et al 2017
	Rsng3	Panama			KF200112.1	Miller et al 2013 (Unpublished)
	Rsng4	Panama			KF200113.1	Miller et al 2013 (Unpublished)
	Rsng5	Portugal	KU556695.1	KU513957.1	KU556746.1	Almeida et al 2017
	Rsng6	Portugal	KU556694.1	KU513956.1	KU556745.1	Almeida et al 2017
	Rsng7	Portugal	KU556693.1	KU513955.1	KU556744.1	Almeida et al 2017
	Rsng8	Portugal	KU556692.1	KU513954.1	KU556743.1	Almeida et al 2017
	Rsng9	Portugal	KU556696.1	KU513958.1		Almeida et al 2017
	Rsng10	Portugal		KU513959.1		Almeida et al 2017
	Rsng11	Portugal		KU513960.1		Almeida et al 2017
	Rsng12	India			KC243872.1	Dantas-Torres et al 2013
	Rsng13	South Africa	KC243786.1	KC243835.1		Dantas-Torres et al 2013
	Rsng14	France	KU255848.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng15	France	KU255849.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng16	Brazil	KC243787.1	KC243836.1	KC243873.1	Dantas-Torres et al 2013

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus sanguineus (Latreille,1806)	Rsng17	South Africa	KC243788.1	KC243837.1	KC243874.1	Dantas-Torres et al 2013
	Rsng18	France	KU255850.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng19	France	KU255851.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng20	South Africa			KC243875.1	Dantas-Torres et al 2013
	Rsng21	Colombia		KC243838.1		Dantas-Torres et al 2013
	Rsng22	France	KC243789.1			Dantas-Torres et al 2013
	Rsng23	USA:Arizona	KU255852.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng24	Honduras			KC243876.1	Dantas-Torres et al 2013
	Rsng25	South Africa	KC243790.1			Dantas-Torres et al 2013
	Rsng26	Honduras			KC243877.1	Dantas-Torres et al 2013
	Rsng27	USA:Arizona	KU255853.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng28	Senegal	KU255854.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng29	Vietnam			KC243878.1	Dantas-Torres et al 2013
	Rsng30	Senegal	KU255855.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng31	Costa Rica			KC243879.1	Dantas-Torres et al 2013
	Rsng32	Senegal	KU255856.1			Rene-Martellet et al 2015 (Unpublished)
	Rsng33	Malta			KX519706.1	Takacs and Farkas 2016 (Unpublished)
	Rsng34	Malta			KX519707.1	Takacs and Farkas 2016 (Unpublished)
	Rsng35	Malta			KX519708.1	Takacs and Farkas 2016 (Unpublished)
	Rsng36	Malta			KX519709.1	Takacs and Farkas 2016 (Unpublished)
	Rsng37	Malta			KX519710.1	Takacs and Farkas 2016 (Unpublished)

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus sanguineus (Latreille,1806)	Rsng38	Malta			KX519711.1	Takacs and Farkas 2016 (Unpublished)
	Rsng39	Malta			KX519712.1	Takacs and Farkas 2016 (Unpublished)
	Rsng40	Malta			KX519713.1	Takacs and Farkas 2016 (Unpublished)
	Rsng41	Cuba	KC018074.1			Sanches et al 2016
	Rsng42	Thailand	KC018075.1			Sanches et al 2016
	Rsng43	USA:Florida		KT382476.1		Zemtsova et al 2016
	Rsng44	USA		KT382477.1		Zemtsova et al 2016
	Rsng45	Canada			KX360403.1	Ondrejicka et al 2017
	Rsng46	Mexico			KX360336.1	Ondrejicka et al 2017
	A8	**Angola**				**This study**
	A9	**Angola**				**This study**
	A13	**Angola**				**Coimbra-Dores et al 2017 (Unpublished)**
	A14	**Angola**				**Coimbra-Dores et al 2017 (Unpublished)**
	CR13	**Portugal**				**Coimbra-Dores et al 2017 (Unpublished)**
	SF1	**Portugal**				**This study**
	P2	**Portugal**				**This study**
	ST4	**Portugal**				**This study**
	ST6	**Portugal**				**This study**
	ST7	**Portugal**				**This study**
	ST8	**Portugal**				**This study**
	CE1	**Portugal**				**This study**
	CE4	**Portugal**				**This study**
	CE7	**Portugal**				**This study**
	CE8	**Portugal**				**This study**
	CE11	**Portugal**				**This study**
	CE12	**Portugal**				**This study**
	CE13	**Portugal**				**This study**

Accession numbers of GenBank sequences used on phylogenetic studies (Continued)

Taxon	Tree Code	Origin	GenBank Accession Number			Bibliographic Reference
			12s	16s	COI	
Rhipicephalus sanguineus (Latreille,1806)	**RI3**	**Portugal**				**This study**
	RI4	**Portugal**				**This study**
	RI5	**Portugal**				**This study**
	RI6	**Portugal**				**This study**
	RI7	**Portugal**				**This study**
	RI9	**Portugal**				**This study**
	RI10	**Portugal**				**This study**
	RI11	**Portugal**				**This study**
	RI12	**Portugal**				**This study**
	RI17	**Portugal**				**This study**
Rhipicephalus spp.	Rsp1	Greece	KC243791.1	KC243839.1	KC243881.1	Dantas-Torres et al 2013
	Rsp2	Greece	KC243792.1	KC243840.1	KC243882.1	Dantas-Torres et al 2013
	Rsp3	Greece	KC243793.1	KC243841.1	KC243883.1	Dantas-Torres et al 2013
	Rsp4	Greece	KC243794.1	KC243842.1	KC243884.1	Dantas-Torres et al 2013
	Rsp5	Spain	KC243802.1	KC243843.1	KC243885.1	Dantas-Torres et al 2013
	Rsp6	Italy	KC243803.1		KC243886.1	Dantas-Torres et al 2013
	Rsp7	Portugal		KC243844.1		Dantas-Torres et al 2013
	Rsp8	Portugal	KC243804.1	KC243845.1	KC243887.1	Dantas-Torres et al 2013
	Rsp9	Portugal	KC243805.1	KC243846.1	KC243888.1	Dantas-Torres et al 2013
	Rsp10	Portugal	KC243806.1	KC243847.1	KC243889.1	Dantas-Torres et al 2013
	Rsp11	Portugal	KC243807.1		KC243890.1	Dantas-Torres et al 2013
	Rsp15	Romania			JX394211.1	Marosi et al 2012 (Unpublished)
	Rsp16	Romania			JX394210.1	Marosi et al 2012 (Unpublished)
	Rsp17	Romania			JX394209.1	Marosi et al 2012 (Unpublished)
	A3	**Angola**				**This study**
	C9	**Cape Verde**				**This study**

9.3 Pathogenic agents' detection

An additional pair of primers were used in order to test the presence of pathogenic agents in some ticks collected from ill hosts, more specifically the EC16S (Cardoso et al., 2016) for Anaplasmatacea family of bacteria in a small sample group.

We have tested five samples for pathogenic agents' detection, RI3, RI4, RI9, RI11 and RI17. Three of this samples have amplified successfully on the ~500bp, suggesting that they were infected with bacteria from Anaplasmataceae family (Fig. 7).

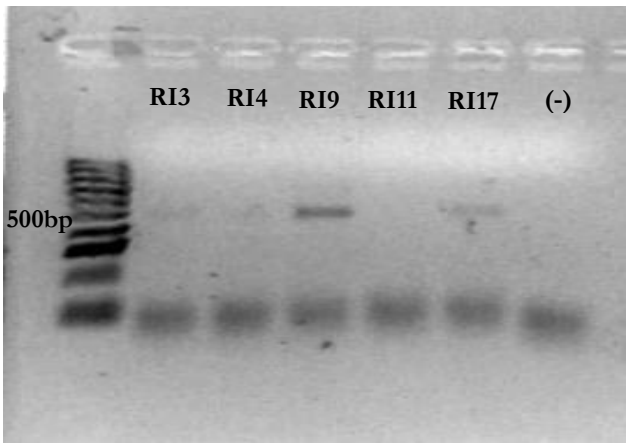

Figure 7: Agarose gel electrophoresis of PCR products (500bp) of positive infected foxes, with Anaplasmataceae family of bacteria (lanes RI3, RI9 and RI17) .The (-) lane is the negative control.

The detection of bacteria from *Anaplasmataceae* family in our samples from ill hosts (foxes), corroborate the information that RIAS provide among the samples information.

Foxes are the most common wild canids in Europe, thus therefore they are a suitable reservoir of TBPs. In recent years, bacteria from the genera *Anaplasma* had received more attention by the scientific community since they were recognized as important human and animal pathogen. *Anaplasma phagocytophilum, A. ovis, and A. bovis* had already been molecularly confirmed to infect foxes from several European countries (Hodžić et al., 2015).

In Portugal, recent studies in this tick-host point out that some other pathogenic agents could be also involved (Cardoso et al., 2015, 2014, 2013).were able to detect *Babesia, Theileria, Hepatozoon canis,* and *Anaplasma platys* in foxes from all over the country. The main vector for this pathogens in Europe are ticks from *R. sanguineus* group. In Portugal, there are a few studies that show the presence of this family of bacteria in ticks based on molecular markers (Maia et al., 2014a; Santos et al., 2009).

All of these diseases have a zoonotic potential and they use to be more associated and reported on domestic dogs, that are reported as the animal that humans have most contact with, increasing the infection risk. (Cardoso et al., 2015; Maia et al., 2014b). Although, due to urbanization and changes on landscape, humans are fragmenting foxes' habitats, so these animals tend to have more proximity with humans and their domestic dogs. For this reason, foxes completed the epidemiological link between domestic dogs and his owners, as describe in Míllan *et al.* (2016). In our study, infected ticks were collected on southern Portugal, corroborating the results obtained by Cardoso *et al.* (2015) who detected *A. platys* in ticks from the same area.